

••••••••••••••••••••••••••••••  
BIRLA CENTRAL LIBRARY  
PILANI (RAJASTHAN)

R

Class No. 616.01

Book No. S989 V-6

Accession No. 59199

••••••••••••••••••••••••••••••





[Crown Copyright Reserved.]



Privy Council  
MEDICAL RESEARCH COUNCIL

A SYSTEM OF  
**BACTERIOLOGY**  
IN RELATION TO  
MEDICINE

VOLUME VI

LONDON :  
PUBLISHED BY HIS MAJESTY'S STATIONERY OFFICE  
1931

## MEDICAL RESEARCH COUNCIL

The Right Hon. the Viscount D'ABERNON, G.C.B., G.C.M.G., LL.D.  
*(Chairman).*

The Right Hon. Lord Mildmay of Flete, P.C. (*Treasurer*).

A. G. CHURCH, D.S.O., M.C., B.Sc., M.P.

Professor J. B. LEATHES, B.M., F.R.C.S., F.R.S.

Professor T. R. ELLIOTT, C.B.E., D.S.O., M.D., F.R.S.

Professor ROBERT MUIR, M.D., Sc.D., F.R.S.

SIR JOHN H. PARSONS, C.B.E., D.Sc., F.R.C.S., F.R.S.

Professor J. J. R. MCLEOD, M.B., D.Sc., F.R.S.

WILFRED TROTTER, M.S., F.R.C.S.

J. A. ARKWRIGHT, M.D., F.R.S.

SIR CHARLES SHERRINGTON, O.M., G.B.E., M.D., Sc.D., F.R.S.

SIR WALTER M. FLETCHER, K.B.E., C.B., M.D., Sc.D., F.R.S.

*(Secretary).*

## BACTERIOLOGICAL COMMITTEE

Professor WILLIAM BULLOCH, M.D., LL.D., F.R.S. (*Chairman*).

SIR FREDERICK W. ANDREWES, D.M., F.R.S.

Captain S. R. DOUGLAS, M.R.C.S., F.R.S., late I.M.S. (*Joint Secretary*).

Professor GEORGES DREYER, C.B.E., M.D., F.R.S.

PAUL FILDES, O.B.E., M.B. (*Joint Secretary*).

W. E. GYE, M.D.

P. P. LAIDLAW, B.Ch., F.R.S.

Professor J. C. G. LEDINGHAM, C.M.G., D.Sc., M.B., F.R.S.

Professor W. W. C. TOPLEY, M.D.

Dr. PAUL FILDES and Professor J. C. G. LEDINGHAM, F.R.S., have acted on behalf of the Committee as general editors of this System of Bacteriology. The chief burdens of its preparation and of other tasks of organization on behalf of the Council have fallen upon Dr. FILDES and Dr. EDGAR SCHUSTER.

For this Volume Professor R. MUIR, Professor C. H. BROWNING, Professor R. T. HEWLETT, Dr. W. M. SCOTT and Professor A. HARDEN have assisted in the editorship with regard to particular sections.

A  
SYSTEM OF BACTERIOLOGY  
.IN RELATION TO MEDICINE

VOLUME VI

BY

J. A. ARKWRIGHT, S. P. BEDSON, C. H. BROWNING, H. R. DEAN,  
A. T. GLENNY, P. HARTLEY, J. C. G. LEDINGHAM,  
H. B. MAITLAND, R. MUIR, H. L. SCHÜTZE, W. M. SCOTT,  
D. B. STEABBEN, J. W. TREVAN



## CONTENTS

	PAGE
<b>CHAPTER I. BACTERIA IN RELATION TO DISEASE</b> .. . . . .	11
THE DISTRIBUTION OF BACTERIA OUTSIDE THE BODY .. . . . .	11
THE VIABILITY OF BACTERIA OUTSIDE THE BODY .. . . . .	11
TRANSMISSION OF BACTERIA TO MAN .. . . . .	14
RESULT OF TRANSMISSION OF BACTERIA TO MAN .. . . . .	15
CONDITIONS GOVERNING THE ESTABLISHMENT OF INFECTION .. . . . .	17
THE BACTERIAL EQUIPMENT TO PRODUCE DISEASE, AND BACTERIAL TOXINS .. . . . .	21
VIRULENCE AND ANTIGENIC COMPLEXITY OF BACTERIA IN RELATION TO EPIDEMIC DISEASE .. . . . .	27
<i>The 'rough' modification, 28 ; The diversity of 'type' characters,             29 ; The antigenic spectrum, 29.</i>	
<b>CHAPTER II. NATURAL IMMUNITY</b> .. . . . .	31
INTRODUCTION .. . . . .	31
THE BASIC DEFENCE MECHANISMS RESIDING IN BODY TISSUES AND FLUIDS .. . . . .	32
<i>Introductory, 32 ; Inflammation, 32 ; Bactericidal elements in blood,             33 ; Opsonins and tropins of normal serum, 36 ; Leukins and             β-lysins, 37 ; The presence in normal mammalian sera of complement-             fixing bodies and agglutinins for various bacteria, 39 ; Functional role             of the antibacterial agglutinins, 39 ; Phagocytosis, 40 ; Lysozyme,             (Fleming), 42.</i>	
INDUCED MODIFICATIONS OF NATURAL RESISTANCE BY PARENTERAL INJECTION OF PROTEINS, COLLOIDS, &c., AND BY VARIOUS PHYSICAL AND CHEMICAL AGENTS .. . . . .	44
<i>The influence of parenteral injection of 'non-specific' substances, 44 ;             Nuclein, deutoeralbumose, starch, &amp;c., 46 ; Ultraviolet rays, 48 ;             Fatigue, 48 ; Conditioned reflexes, 48.</i>	
NUTRITIONAL FACTORS IN RELATION TO PROBLEMS OF NATURAL IMMUNITY <i>Diet and tissue-reactivity to various stimuli, 52 ; Summary, 53.</i>	49
BREEDING EXPERIMENTS IN RELATION TO NATURAL IMMUNITY PROBLEMS <i>Local resistance to toxins, &amp;c., in relation to blood-group inheritance,             54 ; Passive inheritance of antibodies, 55.</i>	53
NATURAL IMMUNITY TO PARTICULAR INFECTIONS OR INTOXICATIONS MET WITH IN CERTAIN ANIMAL SPECIES AND RACES .. . . . .	55
<i>The immunity of the rat to C. diphtheriae and its toxin, 55 ; Natural             resistance of the pigeon to Pneumococcus infection, 57 ; Natural             resistance to anthrax, 57 ; Natural resistance to snake-venom, tetanus             toxin, &amp;c., 59.</i>	
THE PROBLEM OF LOCAL IMMUNITY .. . . . .	60
THE RETICULO-ENDOTHELIAL SYSTEM IN RELATION TO PROBLEMS OF NATURAL IMMUNITY .. . . . .	66
<i>The disposal of organisms introduced into the blood-stream, 67 ; Anti-             body formation, 69 ; Site of antibody formation, 70 ; Blockade of the             reticuloendothelial system and virulence, 71 ; Blockade and chemo-             therapy, 71.</i>	
PHENOMENA ILLUSTRATING THE USE OF 'TISSUE DEPRESSANTS' IN PROMOTING VIRUS OR BACTERIAL ACTION .. . . . .	72

## CONTENTS

	PAGE
<b>CHAPTER III. THE PRODUCTION OF ACTIVE IMMUNITY TO BACTERIAL AND VIRUS INFECTIONS OF MAN AND ANIMALS .. . . . .</b>	<b>77</b>
<b>INTRODUCTION .. . . . .</b>	<b>77</b>
<b>METHODS OF IMMUNIZATION WHICH HAVE BEEN EMPLOYED AT VARIOUS PERIODS AND CRITERIA OF ASSESSMENT .. . . . .</b>	<b>78</b>
The Pre-Jennerian and Jennerian periods. Variolation and vaccination	78
The contributions of Pasteur. Exploitation of 'attenuated' virus (fowl cholera, anthrax, swine erysipelas, rabies) .. . . . .	80
Modifications or amplifications of the Pasteurian method which are still current in veterinary disease control. Also some applications, no longer current, to bacterial diseases of man (typhoid, cholera, plague)	82
Post-Pasteurian period. Introduction of the 'killed' antigen as the immunizing agent. Application to typhoid, cholera, plague, &c., and certain virus-diseases of animals .. . . . .	84
Oral methods of immunization .. . . . .	96
<b>ACTIVE IMMUNIZATION IN THE LIGHT OF RECENTLY ACQUIRED KNOW- LEDGE OF BACTERIAL ANTIGEN .. . . . .</b>	<b>98</b>
<b>CONCLUDING REMARKS .. . . . .</b>	<b>103</b>
 <b>CHAPTER IV. ACTIVE IMMUNIZATION WITH TOXIN .. . . . .</b>	<b>106</b>
<b>METHODS OF TESTING TOXIN AND UNITS OF MEASUREMENT .. . . . .</b>	<b>106</b>
Definitions of units .. . . . .	107
Tests for toxicity .. . . . .	108
Tests for combining power .. . . . .	111
<i>Subcutaneous method</i> , 111; <i>Intracutaneous method</i> , 112; <i>In-vitro method</i> , 115.	
<b>THE ACTION OF CHEMICAL AND PHYSICAL AGENTS UPON TOXIN .. . . . .</b>	<b>122</b>
Toxoid formation .. . . . .	123
<i>The action of formaldehyde on toxin</i> , 124; <i>Other toxoiding agents</i> , 126.	
Modified toxin, other than toxoid .. . . . .	126
The action of phenol on toxin .. . . . .	127
Precipitation of toxins by salts .. . . . .	128
Precipitation of toxin by acid .. . . . .	129
The action of heat on toxin .. . . . .	131
Freezing of toxin .. . . . .	132
Stability of toxin .. . . . .	132
Shaking of toxins .. . . . .	133
<b>ACTION OF CHEMICAL AND PHYSICAL AGENTS ON ANTITOXIN .. . . . .</b>	<b>133</b>
<i>Stability of antitoxin</i> , 134.	
<b>THE ACTION OF CHEMICAL AND PHYSICAL AGENTS UPON TOXIN-ANTITOXIN MIXTURES .. . . . .</b>	<b>135</b>
<b>COMBINATION BETWEEN TOXIN AND ANTITOXIN .. . . . .</b>	<b>136</b>
Modification of toxin .. . . . .	136
<i>Toxoid</i> , 136; <i>Toxone</i> , 138.	
Partial saturation of toxin by antitoxin .. . . . .	139
Relative affinity of toxin and toxoid for antitoxin .. . . . .	140
<i>Relation between units of combining power</i> , 142.	
Accuracy of testing for m.l.d. and L+ .. . . . .	145
Danysz phenomenon .. . . . .	147
Interaction of antigen and antibody .. . . . .	149
Avidity .. . . . .	150

## CONTENTS

7

	PAGE
<b>IMMUNITY, ANTIGENS AND IMMUNITY RESPONSE . . . . .</b>	152
Natural immunity . . . . .	152
<i>Passively acquired natural immunity, 152; Actively acquired natural immunity, 153.</i>	152
Passive immunity . . . . .	155
<i>Method of administration of the antibody, 155; Duration of passive immunity, 157.</i>	155
Active immunity . . . . .	158
<i>Primary stimulus, 160; Secondary stimulus, 161; General principles of immunity response, 162.</i>	158
Methods of testing antigens . . . . .	172
<i>Comparison between methods of testing antigenic efficiency, 176.</i>	172
Diphtheria prophylactic . . . . .	178
<i>Toxin-antitoxin mixtures—Behring's T.A., 179; Toxin-antitoxin mixtures—T.A.M. 3 L+, 179; Toxin-antitoxin mixtures—T.A.M. 1/10 L+, 179; Toxoid-antitoxin mixtures, 180; Toxoid, 181; Toxin-antitoxin floccules, 182; Toxoid-antitoxin floccules, 183; Sodium ricinoleate toxin or toxoid, 183.</i>	178
<b>PRODUCTION OF ANTITOXIN . . . . .</b>	183
Choice of horse . . . . .	183
Choice of toxin . . . . .	185
Course of immunization . . . . .	187
<b>CHAPTER V. THE PHYSIOLOGICAL ACTION OF SOME TOXINS . . . . .</b>	194
<b>CHAPTER VI. GENERAL PROPERTIES OF ANTIGENS AND ANTIBODIES . . . . .</b>	199
INTRODUCTION . . . . .	199
ANTIGENS AND ANTIBODIES . . . . .	202
<i>Conditions of antigenic action, 205; Recognition of antibodies, 207; Phenomena of combination of antigen and antibody, 209; Conditions affecting the development of antibodies, 211; Nature of antibodies and theories regarding their origin, 213; The antigenic properties of receptors combined with antibody, 216; Specificity, 217; Characters of antibodies depending on their source, 219; Multiplicity of antibodies: avidity, 220.</i>	202
<b>CHAPTER VII. THE EFFECT OF PHYSICAL AND CHEMICAL AGENCIES ON THE PROPERTIES OF ANTIGENS AND ANTIBODIES . . . . .</b>	224
ANTIGENS . . . . .	224
The effect of denaturation and coagulation on antigenic function . . . . .	224
The effect of alkalis on antigenic function—racemization . . . . .	228
The effect of hydrolysis of proteins on antigenic function . . . . .	230
Chemical structure and specificity . . . . .	232
ANTIBODIES . . . . .	249
The influence of physical and chemical agencies on the activity of antibodies . . . . .	250
Desiccation, 250; Heat, 251; Light, 252; Hydrolysis by enzymes, 253; Alcohol, 253; The effect of other chemical substances—antiseptics and preservatives, 254.	250
The distribution of antibodies in antisera . . . . .	255
Comparison of the chemical and physical properties of normal sera and antisera, and of the globulins of these sera . . . . .	257
The increase in globulin in the serum of immunized animals . . . . .	258
The purification and concentration of antisera . . . . .	259
Attempts at the isolation of antibodies . . . . .	262

## CONTENTS

	PAGE
<b>CHAPTER VIII. BACTERICIDAL ACTION</b>	267
<b>ANTIBACTERIAL SERA</b>	267
<b>BACTERICIDAL ACTION</b>	269
<i>Pfeiffer's phenomenon—bacteriolysis, 270 ; Anti-immune-bodies, 273 ; Deviation of complement : Neisser-Wechsberg Phenomenon, 274 ; Intensification of bacteriolysis, 276 ; Dissociation of immune-body, 277 ; Variety of receptors, 277 ; Receptors and virulence, 278 ; Relations of bactericidins to other antibodies, 279 ; The relations between different types of immune-body, 280 ; Bacteriolysis in relation to antitoxic action, 282 ; Normal bactericidal action, 283 ; Bactericidal action apart from complement, 284.</i>	
<b>GENERAL CONSIDERATIONS</b>	287
<b>ANTIAGGRESSINS</b>	290
<b>CHAPTER IX. HÆMOLYTIC ACTION</b>	295
<b>INTRODUCTION</b>	295
<b>NATURE OF LYSIS</b>	296
<b>CONDITIONS AFFECTING LYSIS</b>	296
<b>HÆMOLYTIC RECEPTORS</b>	300
<b>THE UNION OF IMMUNE-BODY</b>	301
<b>DISSOCIATION OF IMMUNE-BODY</b>	302
<b>SPECIFICITY OF IMMUNE-BODY</b>	305
<b>QUALITATIVE VARIATION IN IMMUNE-BODY</b>	306
<b>ISOLYSINS</b>	307
<i>The Question of Anti-Immune-Bodies, 308</i>	
<i>The Nature and Mode of Action of Immune-Body, 310</i>	
<i>Natural Immune-Bodies, 313</i>	
<i>The Combination and Lytic Action of Complement, 314</i>	
<i>Forssman's Heterophile Antibody, 315</i>	
<b>CYTOLYSINS</b>	322
<i>Nature of cytolsins, 323</i>	
<i>Specificity of cytolsins, 323</i>	
<i>Isocytolsins, 325</i>	
<i>Autocytolsins, 325</i>	
<i>Anticytolsins, 326</i>	
<i>Review of the various cytolytic sera which have been studied</i>	
<i>Anticell sera, 326 ; Anti-organ sera, 330.</i>	326
<b>CHAPTER X. COMPLEMENT</b>	332
<b>INTRODUCTION</b>	332
<b>DEFINITION AND GENERAL PROPERTIES</b>	332
<b>COMPLEMENT-FIXATION</b>	335
<b>DEVIABILITY</b>	338
<b>THE 'MULTIPLICITY' OF COMPLEMENTS</b>	339
<b>CONSTITUTION OF COMPLEMENT ESPECIALLY IN RELATION TO SERUM-PROTEINS</b>	339
<b>INFLUENCE OF PHYSICAL AND CHEMICAL AGENTS</b>	343
<b>FUNCTIONS, MODE OF ACTION, ORIGIN AND NATURE OF COMPLEMENT</b>	346
<i>Functions, 346 ; Mode of action, 347 ; Origin, 348 ; Nature, 349.</i>	
<b>CHAPTER XI. OPSONIC ACTION : TROPINS</b>	353
<b>INTRODUCTION</b>	353
<b>OPSONIC ACTION OF NORMAL SERUM</b>	356
<i>The question of the specificity of normal opsonic action, 356</i>	
<i>Complement and opsonic action, 357</i>	
<i>The complementing of natural immune-bodies, 360</i>	
<i>Normal tropins, 362</i>	
<i>Non-specific opsonic action of complement, 363</i>	
<i>Spontaneous phagocytosis, 363</i>	
<i>Opsonic action on inert particles, 364</i>	
<i>Distribution of opsonic substances, 365</i>	
<i>Inhibition of phagocytosis, 365</i>	365

## CONTENTS

9

	PAGE
<b>IMMUNE SERA . . . . .</b>	<b>366</b>
Bacteriotropins . . . . .	366
Immune-bodies in opsonic action . . . . .	368
<b>PHAGOCYTOSIS IN VIVO AND IN VITRO . . . . .</b>	<b>372</b>
<b>INTRACELLULAR AND EXTRACELLULAR BACTERICIDAL ACTION . . . . .</b>	<b>374</b>
<b>MODE OF ACTION OF OPSONIC SUBSTANCES . . . . .</b>	<b>376</b>
<b>CHAPTER XII. AGGLUTINATION . . . . .</b>	<b>381</b>
<b>INTRODUCTION . . . . .</b>	<b>381</b>
<b>AGGLUTINATION OF BACTERIA: SPECIFIC AND NON-SPECIFIC . . . . .</b>	<b>382</b>
Surface charge or difference of potential (P.D.) between the particles and the suspending liquid . . . . .	382
Agglutination of bacteria by cohesion of the different surfaces which they present . . . . .	383
Coagulation of interbacillary substance . . . . .	384
<b>NON-SPECIFIC AGGLUTINATION OF BACTERIA IN THE ABSENCE OF SPECIFIC SERA . . . . .</b>	<b>384</b>
The effect of neutral salts on somatic agglutination in the absence of agglutinins . . . . .	384
<i>Relation of P.D. to non-specific agglutination by salts, 385; The effect of certain additions to suspensions on their agglutinability by salt, 386.</i>	
Agglutination by acid . . . . .	386
<i>Flagellar agglutination, 386; Somatic agglutination by acid, 387; Effect of heating the suspension, 387; Cataphoresis, 388; Effect on acid agglutination of additions to bacterial emulsions, 388.</i>	
<b>SPECIFIC SERUM AGGLUTINATION . . . . .</b>	<b>389</b>
Agglutinins . . . . .	389
Agglutinogens, antigens and agglutinable substances . . . . .	390
The mechanism of agglutination . . . . .	391
Combination of agglutinin and agglutinogen . . . . .	393
The stage of clumping . . . . .	393
Thread reaction . . . . .	394
The different kinds of agglutinogen and agglutinin . . . . .	394
Precipitation . . . . .	400
<i>Flagellar antigen, 400; Somatic antigen and endotoxin, 400; Chemically prepared fractions of antigens, 400; Exotoxin, 403.</i>	
Multiple flagellar and somatic antigens . . . . .	403
<i>Qualitative serological analysis of the antigenic complex, 403; Quantitative agglutination, 406; The practical value of qualitative analysis, 408.</i>	
Bacterial suspensions for agglutination . . . . .	411
<i>Medium for suspending the bacteria, 412; Living, killed and preserved suspensions of bacteria, 413; Preservation of serum, 413.</i>	
Normal agglutinins . . . . .	413
Paragglutination . . . . .	414
The production of agglutinins as the result of infection or vaccination . . . . .	414
The value of agglutination as an aid to clinical diagnosis . . . . .	415
General importance of the agglutination reaction: relation of agglutinogens to virulence and to immunizing capacity . . . . .	416
Agglutination as a guide to classification . . . . .	418
<b>CHAPTER XIII. THE PRECIPITATION REACTION . . . . .</b>	<b>424</b>
<b>INTRODUCTION . . . . .</b>	<b>424</b>
<b>PREPARATION OF PRECIPITATING SERA . . . . .</b>	<b>425</b>
<b>METHODS OF PERFORMING THE PRECIPITATION REACTION . . . . .</b>	<b>425</b>
<b>THE INFLUENCE OF OPTIMAL PROPORTIONS OF ANTIGEN AND ANTIBODY IN THE REACTION . . . . .</b>	<b>426</b>
<b>TITRATION OF ANTISERUM BY THE OPTIMAL PROPORTIONS METHOD . . . . .</b>	<b>428</b>
<b>THE INFLUENCE OF REACTION, TEMPERATURE, SALTS AND DILUTION . . . . .</b>	<b>430</b>
<b>SOURCE OF THE PRECIPITATE . . . . .</b>	<b>432</b>

## CONTENTS

	PAGE
SPECIFICITY AND PRACTICAL APPLICATIONS .. . . . .	437
THE RELATION OF PRECIPITATION TO OTHER SERUM REACTIONS .. .	441
THE RELATION BETWEEN THE FIXATION OF COMPLEMENT AND THE FORMATION OF A PRECIPITATE .. . . . .	442
AGGLUTINATION AND PHAGOCYTOSIS .. . . . .	448
<b>CHAPTER XIV. ANTIFERMENTS AND DEFENSIVE FERMENTS</b> .. . . . .	<b>452</b>
ANTIFERMENTS .. . . . .	452
DEFENSIVE FERMENTS—ABDERHALDEN'S REACTION .. . . . .	453
<b>CHAPTER XV. ANAPHYLAXIS AND RELATED PHENOMENA</b> .. . . . .	<b>457</b>
ANAPHYLAXIS .. . . . .	457
Introduction .. . . . .	457
Definition .. . . . .	457
Symptoms and lesions of anaphylaxis .. . . . .	458
<i>In the guinea-pig, 458 ; In the dog, 459 ; In the rabbit, 460 ; In other animals, 461 ; Blood changes, 462 ; Essential reactions, 462.</i>	
Local anaphylaxis .. . . . .	462
Sensitization .. . . . .	463
<i>Anaphylactogens, 463 ; The anaphylactic antibody, 465 ; Inheritance of anaphylactic sensitiveness, 467 ; Combining relationships of anaphylactogen and anaphylactic antibody, 468 ; Reversed anaphylaxis, 468.</i>	
Anti-anaphylaxis .. . . . .	469
Anaphylactoid reactions .. . . . .	472
<i>Cellular anaphylaxis, 472 ; Cytotoxic sera, 473 ; Anaphylatoxin or serotoxin, 474 ; Peptone shock, 475 ; Histamine shock, 476.</i>	
The cause of anaphylaxis .. . . . .	478
<i>The histamine hypothesis, 480.</i>	
SERUM-SICKNESS .. . . . .	481
Symptoms and incidence .. . . . .	481
Cause of serum-sickness .. . . . .	482
Practical considerations .. . . . .	483
<b>ALLERGY: ITS RELATION TO ANAPHYLAXIS</b> .. . . . .	<b>483</b>
<b>IDIOSYNCRASIES</b> .. . . . .	<b>485</b>
Definition, incidence, heredity .. . . . .	485
Passive transference .. . . . .	486
Mechanism .. . . . .	487
<b>CHAPTER XVI. THE BEARING OF COLLOIDAL CHEMISTRY UPON SOME IMMUNITY REACTIONS</b> .. . . . .	<b>490</b>
<i>Introduction, 490 ; Surface tension, 491 ; Flocculation of colloids, 493 ; Filtration, 494 ; Hydrogen-ion concentration, 495 ; Viscosity, 495 ; Permeability, 496 ; Dialysis, 496 ; Denaturation, 497 ; Adsorption, 497.</i>	
<b>CHAPTER XVII. CHEMOTHERAPY</b> .. . . . .	<b>501</b>
INTRODUCTION .. . . . .	501
INFECTIONS WITH TRYPANOSOMES .. . . . .	501
<i>Drug-resistance, 505 ; Chemoflexion, 508 ; Combined therapy, 509 ; Therapeutic interference, 509 ; Immunity following treatment with chemotherapeutic agents : serum-resistance, 510.</i>	
INFECTIONS WITH SPIROCHÄTES .. . . . .	512
THE MODE OF ACTION OF CHEMOTHERAPEUTIC AGENTS IN INFECTIONS WITH TRYPANOSOMES AND SPIROCHÄTES: EHRLICH'S RECEPTOR THEORY .. . . . .	512
MALARIA .. . . . .	516
PIROPLASMOSIS .. . . . .	519
AMÆBIC DYSENTERY .. . . . .	522
BACTERIAL INFECTIONS .. . . . .	523
<i>Antiseptics, 523 ; Metal salt therapy, 527.</i>	
GENERAL CONSIDERATIONS .. . . . .	529

## CHAPTER I. BACTERIA IN RELATION TO DISEASE.

BY H. B. MAITLAND (UNIVERSITY OF MANCHESTER) ;  
WITH A SECTION BY  
W. M. SCOTT (MINISTRY OF HEALTH).

### The Distribution of Bacteria Outside the Body.

PATHOGENIC bacteria are, in general, those which are so essentially parasitic that they cannot survive indefinitely outside the body under natural conditions ; their continued existence depends upon their ability to resist natural destructive agencies during the intervals between their propagation in a suitable host, although the host need not necessarily be infected. The peculiarities of individual species in respect of the type of disease they cause, or their saprophytic existence in a host, as for example the anaerobes in the intestine of animals and man, and their viability outside the body, determine their distribution in nature. Complementary to this, the distribution of different species appears to be that which is suitable for their re-entry into a host in a manner favourable to their continued propagation. In illustration, one may refer to the distribution of pathogenic anaerobes in soil, of enteric organisms in water, of the bacteria which cause respiratory infections in droplets and dust. The conception that in the course of time a process of parasitic adaptation has taken place and resulted in the establishment of the pathogenic bacterial species in their present form would take into account the reaction between bacteria and host, the manner of extrusion of bacteria from the host, their viability and distribution in nature as essential parts of this process. Experimentally, we are aware of adaptation to different hosts, of variations in virulence and pathogenicity, of factors in the resistance of the host peculiar to groups, but the possible effects of these and other agencies in the slow establishment of bacterial species in their present state can only be surmised. The matter is discussed in relation to the *Salmonella* group in Vol. IV, p. 110 ; see also Chapter II. The adoption of the point of view that they have resulted from a process of parasitic adaptation tends, however, to place the known facts of distribution in more significant relation to disease considered as a biological phenomenon.

### The Viability of Bacteria Outside the Body.

*Drying.* Lange and Jochimsen (1927) have discussed the general considerations to be taken into account in assessing the importance of dust as a vehicle for transmitting infection, and have reviewed the data upon which present opinion has been founded. It has been held that many bacteria will not survive the drying which is required before sputum,

for example, will flake off in particles fine enough to be carried readily in the air. Material containing mucus or protein tends to adhere to clothing or other objects on which it dries and is not easily dislodged except by vigorous mechanical means. The particles which come off are usually too large to be carried far in the air and are deposited. The finer and more dangerous particles are composed of inorganic matter or are small fibres from cloth. These are more likely to be inhaled since they remain longer in the air. Köhlisch (1908) has estimated that only 4 per cent. of bacteria inhaled with dust reach the lungs. *V. cholerae*, *B. pestis*, *Pneumococcus*, *Meningococcus*, *B. influenzae*, *B. pertussis* and *C. diphtheriae* have been included among the bacteria which would not withstand the drying necessary to give rise to fine particles. They are therefore not readily transmitted by dust. For more resistant bacteria, for example *B. tuberculosis*, transmission by dust has been held to be less important than droplet infection.

Lange, as a result of a series of experiments, believes that this point of view is an underestimate of the importance of dust in transmitting infection. Lange and Jochimsen (1927) have found that *B. influenzae*, which has comparatively little resistance to drying, can be carried in fine dust and may be transmitted for short distances. It was possible for viable *B. influenzae* and *B. prodigiosus* in dried sputum to be given off for a short time from clothing during ordinary movement; 12 per cent. of infected particles shaken from infected material dried on handkerchiefs remained in the air for 30 minutes. Dust from clothing was more dangerous than that from floors; only 1 per cent. of infected particles from the latter source remained in the air for half an hour.

The fate of *B. tuberculosis* in dried sputum has been reinvestigated by Lange (1926), who found that there may be no loss of viability during the first 24 hours even when sputum was dried in thin layers. The dust produced by brushing and beating soiled clothes or soiled dusty boards produced infection in 25 per cent. of guinea-pigs which were made to breathe it, and the lesion first appeared in the lungs. The experiments of Jochimsen (1928) indicated that *C. diphtheriae* could also be carried in dust.

*Light.* The effect of light on bacteria under natural conditions can be stated only in general terms. The concurrent effects of the medium which surrounds the bacteria, the temperature and state of moisture, influence the rate of destruction. But under controlled experiment relevant information has been obtained. Barnard and Morgan (1903) determined that the radiations which destroyed bacteria lay between the wave-lengths 3,287 and 2,265 A.U., about the middle third of the ultra-violet as seen in a photograph of the spectrum of carbon. Four-fifths of these rays were absorbed by a layer of water 2·5 cm. thick. The action of the remainder of the spectrum was negligible in comparison, although probably there is a slight activity throughout the whole of it. The effect of light varied in direct proportion to its intensity. These limits of wave-lengths have been confirmed by others, including Browning and

Russ (1919), and Bayne-Jones and Van der Lingen (1923). The latter authors observed that the limits of effective wave-lengths coincided with the absorption of ultraviolet by bacteria, from 3,500 A.U. to 1,856 A.U. As the wave-length decreased the bactericidal intensity increased. The effect of temperature was small, the temperature coefficient being 1·05 over a range of 10° C. The reaction of the suspending medium markedly influenced the time which the lethal rays required to kill bacteria. At reactions more acid than the isoelectric point acceleration in the rate of the bactericidal action was marked. The actual times required to kill bacteria exposed directly to light are short. No significant differences were found between Gram-positive, Gram-negative and acid-fast organisms by Bayne-Jones and Van der Lingen. Burge and Neill (1915) tested seven strains of Gram-positive and Gram-negative bacteria by exposing emulsions in quartz tubes and found that from 120 to 200 seconds sufficed. Fluorescent bacteria survived exposure for 200 seconds and appeared to be more resistant.

In nature the destructive action of sunlight will depend upon the intensity of ultraviolet radiation. Temperature will have little influence, but the material surrounding the bacteria will be an important factor in determining the result, both as regards its reaction and its ability to offer protection by absorbing radiation. It is of interest also that different species (apart from fluorescent bacteria) appear to be about equally susceptible.

*Spores.* The spore is a normal stage in the life-cycle of bacteria, and represents a phase of bacterial life in which the processes of a living cell though slowed are not absent. The spore undergoes slow but continuous alterations, including alterations in resistance. It is probable that spores of different species do not behave alike, but the work of Magoon (1926) on spores of *B. mycoides* may be taken as an indication of principles which may apply generally. Testing resistance to heat he found that the age of spores and the conditions of moisture and temperature in which they had been kept influenced the result. Changes in resistance took place more slowly when the spores were kept dry and cold, the highest resistance developed under moderate temperature and humidity. In most of the experiments there was a period of maximum resistance which varied with the environment. It is therefore to be expected that the resistance of spores to other destructive agents may increase for a time after they are freed from the host and that the effect of environment on this phase of the life of the spore may determine the duration of its viability. Spores developed in cultures and presumably also those formed in a host are not individually alike, but vary in resistance, which in part depends on their maturity. Swann (1924) noted that about 5 per cent. of anthrax spores from cultures up to one week old were dead as compared with about 55 per cent. dead in agar cultures which had been dried over calcium chloride for one year. Such individual differences have been evident in tests of resistance, and may be held to apply to viability under natural

conditions. Heat, and by analogy possibly other destructive agents also, may increase the time required for the germination of spores. This has been shown experimentally for spores of *B. botulinus* by Dickson, Burke, Beck and Johnston (1925). Swann (1924) found that anthrax spores germinated more slowly when they were old and dried than when they were young and moist. The relation of dormancy to infection is only suggested by these results, but it is recognized that the spores of anaerobes may persist in war wounds for many years. The case recorded by Pulvertaft (1929) is an instance.

*Water, soil, milk.* The survival of bacteria in water, soil and milk is treated in Vol. III; the distribution of bacteria of the enteric and *Salmonella* groups in nature is discussed in Vol. IV.

### Transmission of Bacteria to Man.

The opportunities for transmission of bacteria to the human host are often determined by circumstances peculiar to our manner of living. The assembly of people in halls, trams and trains, the contact afforded in schools, overcrowding in slums, the collective distribution of milk and water, and other similar circumstances favourable to transmission have no doubt affected the incidence of some infectious diseases, have accounted for outbreaks, and have influenced the spread of epidemics. Opportunity for transmission does not, however, always favour the establishment of infection. The resistance of prospective hosts, individually or collectively, may be increased by the reception of repeated amounts of bacteria too small to set up infection, as a result of continued residence in an environment where opportunity for bacterial transmission is favourable. This possibility was recognized for diphtheria many years ago (Wassermann, 1895), and evidence confirming it followed the introduction of the Schick test (Medical Research Council, 1923). The recent work of Topley (1926) and others on experimental epidemics in animals and of Dudley (1929) on diphtheria in a residential school community is important in this connection. This process, which may be said to favour the host, appears to apply chiefly to certain infections which gain entrance to the body by the respiratory tract and to some which enter by the alimentary canal. On occasions the circumstances permitting the transmission of bacteria put the host at a distinct disadvantage. These are comparatively sudden occurrences, where the host is subjected to unusual exposure as, for example, outbreaks of meningitis (a relatively uncommon infection in civil life) when recruits enter camp, gas gangrene and tetanus infections in wounds, milk-borne infections due to streptococci or other bacteria, water-borne intestinal infections, exposure of industrial workers to anthrax, the introduction of diphtheria or other infections into schools.

There appears to be a necessary relationship between the method of transmission of individual species of bacteria and their habitat and viability, which meets the requirements of their parasitic adaptation.

*Spirochæta pallida* survives for a very short time outside the body and is transmitted nearly always by direct contact. This applies almost equally to *Gonococcus*. The pathogenic anaerobes survive in soil for long periods before chance permits them to be ingested or to enter a wound. The enteric organisms, coming also from the intestine, are likely to be transmitted more directly in water, milk, &c., than are the anaerobes, and their viability is less than that of these but greater than that of *Meningococcus*, *Pneumococcus*, *B. influenzae* and other bacteria of the respiratory tract which have a direct mode of transmission by droplets. It is, of course, true that these methods do not exhaust the possibilities of transmission by any of the species mentioned, but the general statement is sufficiently significant.

### Result of Transmission of Bacteria to Man.

The transmission of pathogenic bacteria to a suitable host is essential to their continued existence, but the result of transmission depends upon an interaction of factors relating both to host and to parasite which is very complex. In outline the main facts are well known. The result is determined on the one hand by the resistance of the host and on the other by the dose and virulence of the bacteria. It may be recognizable infection with or without a subsequent carrier-state, mild infection which may not be recognizable and may lead to a period during which the host is a carrier, the establishment temporarily of a healthy carrier, or the rapid eradication of the bacteria. Knowledge of the infectious nature of disease has made it possible to prevent the dissemination of bacteria from cases, but the residual carrier in many instances escapes detection and it is not possible to assess his importance in the transmission of some diseases. Of probably more importance are the mild or unrecognized infections and the healthy carriers. It would appear to be an essential provision for maintaining the parasite that the host should not in every instance be destroyed. Epidemics or exacerbations of the incidence of infections are evidence that during interepidemic times the infective agent has been maintained in the population in carriers or in mild or unrecognized cases.

The resistance of many pathogenic bacteria to physical influences is so small that one must regard carriers as the chief means, apart from cases of disease, by which bacteria are maintained in a community and the chief source from which they spread. There cannot be a large permanent reservoir for many species outside the human host. This on *a priori* grounds is evident for such organisms as *Meningococcus*, *Gonococcus*, *Streptococcus*, *Pneumococcus*, *B. pertussis* and *B. influenzae*, *C. diphtheriae* and the enteric group, to mention only the more obvious examples. The possibility of reservoirs in animals must of course always be kept in mind, such as are well recognized for *Br. abortus* and the *Salmonella* group. Our knowledge of viruses does not permit more than an indirect assessment of the importance of carriers for these, but there is every reason to believe

that the carrier is chiefly responsible for maintaining the virus in the community. The difficulty in determining the carrier rate for any particular organism lies in the difficulty in making a sufficient number of examinations of a large enough number of people, and in part also on limitations inherent in methods. A striking illustration of the importance of technique is afforded in the recent paper of Fleming and Maclean (1930), who, by using ' penicilin ' to inhibit other bacteria, demonstrated the presence of *B. influenzae* in the mouth or nasopharynx in 100 per cent. of normal people.

The individual who becomes a carrier as a result of a mild unrecognized infection, and the healthy carrier (the distinction may not always be possible) are most likely to escape detection. They occur in greater numbers among those who have been in contact with cases of disease, for example carriers of *Meningococcus* (Medical Research Council, 1916 and 1917 ; this System, Vol. II, p. 316), of *C. diphtheriae* (Medical Research Council, 1923), of *Pneumococcus* (Avery, Chickering, Cole and Dochez, 1917). The occurrence of healthy carriers of the pathogenic intestinal bacilli is also well recognized, and certain familial outbreaks of dysentery and *Salmonella* infections met with intermittently suggest an origin from carriers who have not been recognized as having had an acute infection. The earlier literature on the importance of healthy carriers in relation to cholera, dysentery, enteric fever and other infections has been reviewed by Ledingham and Arkwright (1912).

That such a state of equilibrium between bacteria and host is possible is due in some measure to immunity developed in the host-population as a result of intermittent exposure to bacteria, through contact with carriers and others. In a community, the balance between carriers, the incidence of disease and the state of immunity of the population collectively (herd immunity) is continually undergoing adjustment. The important studies of Dudley (1929) on diphtheria have shown the interdependence of these processes in a community subjected to accurate observation. That the principles obtained in diphtheria may have wider application is indicated by his reference to poliomyelitis, measles, the common cold and influenza. It should, however, be noted that, as he points out, his attention was confined to infections which are spread chiefly by droplets. Apart from isolation of cases and carriers of some of the diseases spread by this means, nothing has been done by public health measures to alter the natural processes of adjustment between air-borne bacteria and man. With regard to the pathogenic intestinal bacteria, the natural modes of transmission to man have been severely interrupted. Isolation, the control of water supplies, and vaccination to augment resistance have been practised. But in communities of mice, under experimental conditions, Topley (1926) and his colleagues have shown that in mouse paratyphoid infection a similar adjustment goes on. Although the conditions of the experiments were not always such as are likely to happen in human communities as regards exposure to intestinal

infection, the principles established are of the same kind as those demonstrated for other types of infection in human communities and indicate the general importance of herd immunity as a factor influencing the results of transference of bacteria to man.

### Conditions Governing the Establishment of Infection.

That different kinds of bacteria have special requirements for growth in the body is manifested by the susceptibility or insusceptibility of tissues to primary invasion and by the different parts of the body which are involved when infection is established. The explanation of the phenomenon is obscure, but the reason why, for example, *Pneumococcus* and *Meningococcus*, each entering the nasopharynx, characteristically attack different organs must be an essential part of the mechanism by which disease is produced. The explanation cannot be entirely anatomical. It would appear to be governed by the properties of the bacterium in respect to requirements for growth, and to be determined by something more subtle than merely a supply of suitable foodstuff. The work of Rous (1925<sup>1, 2, 3 & 4</sup>) has shown that the pH of different organs varies, and Fildes (1929) has found that the germination of tetanus spores is determined by the eH of the tissue. Voegtlin, Johnson and Dyer (1924) and others have demonstrated differences in the reducing power of normal tissues. These findings may be of wider significance in this connection, and it is possible that more light will be thrown on the problem when the chemical environment of bacteria in tissues has been more thoroughly investigated.

*Local injury.* For a discussion of injury in relation to the invasion of tissues by bacteria or viruses, see Chapter III.

*Selective localization.* The view that different strains within one species have a selective affinity for certain tissues, which has been sponsored chiefly by Rosenow (1928) and applied chiefly to *Streptococci*, implies that there is an environment peculiar to a tissue in which only certain strains can grow, or, conversely, that only some strains are able to overcome the resistance of a certain tissue. The possibility that there may be a basis of truth in this conception cannot be easily disproved ; it is merely the extension of a characteristic possessed by species to strains of the rather diverse group of *Streptococci*. The difficulty is not in recognizing the possibility but in accepting the point of view as fact. The work of Rosenow has not been sufficiently confirmed to enable the majority to concur in his opinion.

*Virulence.* The virulence of bacteria, which may be defined as their ability to invade and multiply in the tissues of a susceptible host, determines, other things being equal, whether or not infection occurs ; the degree of virulence is evidenced by the severity and outcome of the infection. The attempt to understand essentially what change is undergone by bacteria when alterations in virulence take place has resulted in

the problem being approached from several sides, and it is by the correlation of work on bacterial variation, on the chemical analysis of antigens, and on experimental epidemiology that the meaning of virulence is beginning to be understood. A valuable summary of the problem has been made by Arkwright (1929), particularly of the knowledge that has come from a study of variation (see also Vol. I, p. 339). The relation of virulence to epidemiology is dealt with on p. 27.

The pathogenicity of bacteria which produce exotoxin appears in some species to be a function of that property. Non-toxic variants of *B. tetani* and *C. diphtheriae* are non-pathogenic. The question as to whether the severity of infection caused by the toxin-producing strains depends only upon the amount of toxin which is formed requires further investigation. It has to be decided for each species whether in addition to toxin-production there is a property of virulence associated with the bacterial cell itself. It has been shown by Downie (1930), working with *Streptococcus haemolyticus*, that, although the presence of toxin at the site of inoculation can determine a fatal infection through its action in enabling the bacteria to gain an initial advantage in invading the tissues, strains of widely differing degrees of invasive power produce *in vitro* approximately the same amount of toxin as judged by skin-tests on man. In this instance virulence appears to be a function of some property of the organism other than its power to produce toxin. For those species which do not spread widely in tissues, e.g. *C. diphtheriae*, the degree of virulence may appear to accord more closely with toxigenicity. The apparent relation of virulence and toxigenicity is that in some species the presence of toxin at the site of primary contact of bacteria with tissue repels the leucocytes and protects the bacteria from phagocytosis, thus giving them an opportunity to establish themselves. This does not necessarily indicate, however, a property of virulence. With invasive species the virulent organisms have a greater capacity for multiplication in tissues, or are more able to resist phagocytosis or other destructive agencies. This capacity for growth in tissues may, as Arkwright (1929) points out, be the true meaning of virulence.

A distinct advance in knowledge has been made by the antigenic analysis of bacterial variants in relation to their virulence. The so-called rough (R) non-virulent and the smooth (S) virulent forms of a single strain are distinguished, among other differences, by the nature of their somatic or heat-stable antigens. This difference in antigenic composition appears to be responsible for the difference in virulence characteristic of these forms. The meaning of antigenic specificity in terms of chemistry has begun to be appreciated from the work on the chemical analysis of antigens (Landsteiner, 1928) (see Chapter VII). In *Pneumococcus* (Avery and Heidelberger, 1925) the specific serological characters of the several types depend upon the chemical structure of a polysaccharide (the so-called 'soluble specific substance') which in the cell is united to protein. This substance is specially abundant in the capsules and is absent in the

rough avirulent forms of *Pneumococci* which typically have no capsule. A similar compound has been isolated from Friedländer's bacillus (Avery, Heidelberger and Goebel, 1925), and Julianelle (1926) has shown that it is responsible for type-specificity, and is present only in the smooth forms, which possess capsules and are of exalted virulence. The well-known association of virulence and capsules appears to depend on the large amount of soluble specific substance which the capsule contains. How this substance operates to effect virulence is not understood; detached from protein it is not toxic, nor does it stimulate the formation of antibodies. It does, however, unite with antibodies, and the association of capsule and virulence may mean that the polysaccharide in the capsule absorbs the antibodies and prevents them from acting upon the vital part of the cell. The protective function of a capsule may therefore be ascribed to its chemical composition as well as to its physical properties, which in themselves are believed to make phagocytosis difficult. Whether the capsule by virtue of its content of soluble specific substance assists the bacterium to multiply in the tissues of a normal host is not certain. It is possible that there may be an effect of this kind and that certain chemical compounds may function to produce virulence both by protection and by assisting growth.

That the possession of a capsule is not the universal explanation of virulence is evident from work on *Streptococci*, for example. Certain highly virulent strains (McLeod, 1915; Downie, 1930) which are non-capsulated will kill mice in doses of 0·000,001 c.cm. and rabbits in doses of 0·01 c.cm. of culture. With such strains virulence is related to the ability of the strain to proliferate in the serum of the test animal, and it would be of great interest to know to what extent correlation between content of soluble specific substance, ability to grow in serum or tissue, and virulence, may apply generally both for capsulated and non-capsulated species. An accession of virulence by repeated passage of a culture through a susceptible host does not necessarily mean that the bacteria have acquired a greater amount of this substance, although there is evidence that this may be possible. The fact has to be kept in mind that a culture usually consists of a mixture of bacteria of varied degrees of virulence and that repeated passage is a process in which the more virulent individuals become predominant. The experiments of Griffith (1928), which have been confirmed by Neufeld and Levinthal (1928), Reimann (1929) and Dawson (1930<sup>1 & 2</sup>), indicate, however, that the soluble specific substance can be acquired *in vivo*. Mice were injected subcutaneously with a rough avirulent strain of *Pneumococcus* and a culture of a smooth virulent strain of another type which had been killed by heat. The mice died of *Pneumococcus* infection, and the organism which was recovered was in a fair number of instances a capsulated strain of the type of the killed culture. It is still not clear in what way changes of this kind can occur in nature. But it is known that the presence of serum tends to maintain the virulence of cultures, while on the other hand the cultivation of smooth forms in

antismooth serum has been shown to accelerate the change to the rough form. There is thus suggestive evidence that alterations in virulence may take place *in vivo* apart from a process of selection, due to gain or loss of soluble specific substance.

The extreme virulence of bacteria taken direct from infected animals has been studied with accuracy for *Streptococcus* and *B. anthracis*. Using a micro-pipette and dark-ground illumination (Péterfi and Wámoscher, 1926) it was possible to isolate and infect with a single bacterium. Marx (1927), working with the highly virulent strain 'Aronson' of *Streptococcus haemolyticus*, observed that one organism from the spleen of an infected mouse, inoculated into the tissues of the chest wall, caused a fatal infection in 32 per cent. of white mice. The mortality was 100 per cent. when more than 10 streptococci were inoculated. Similarly, Martos (1927), using a highly virulent strain of *B. anthracis*, observed that the inoculation of one bacillus produced a fatal infection in 28 per cent. of mice and that 100 per cent. died when 10 to 20 bacilli were inoculated. These experiments were made with admittedly highly virulent strains but suggest that the individual bacteria of these strains obtained from infected tissue possessed high virulence. It may be noted that Wámoscher (1926) obtained similar results with a highly virulent culture of Type III *Pneumococcus*; a single organism produced 23 per cent. of fatal infections in mice.

*Nutrition of the host.* The state of nutrition of an animal in determining the character or extent of tissue reaction to bacteria or toxins is a subject of much interest, which is discussed more fully in Chapter III. It is sufficient to mention here that the inflammatory reaction may be markedly altered as a result of the state of nutrition. It has been mentioned (Vol. VII, p. 61) that young guinea-pigs or older animals not gaining in weight do not react well to foot-and-mouth disease virus. The local lesions are likely to be small and to develop slowly, and generalized lesions may not appear. A similar observation has been made by Ford and Eliot (1928) in transferring *Bartonella muris* to rats, though their experiments did not involve the development of local lesions.

Decrease in the inflammatory reaction has also been produced experimentally by reducing the amount of a balanced diet or by deprivation of vitamin (Arkwright and Zilva, 1924). The failure to produce a vigorous inflammatory response may assist the invasion of bacteria and affect unfavourably the outcome of infection (Schütze and Zilva, 1927). On the other hand, in diseases which are not usually fatal, such as foot-and-mouth disease, the severity of infection is evidenced by the severity of the lesion. Animals which do not react vigorously appear to have a mild attack. In foot-and-mouth disease it would seem that the serous inflammatory exudate favours the growth of virus in the local lesion, for the richest yields of virus are obtained from vesicles which are large and develop rapidly.

*Allergy.* In contrast to the occurrence of decreased inflammatory reaction, there is, connected with the allergic state, an accentuation of the

inflammatory response which tends towards increased resistance of the host. The classical example is the lesion which follows the inoculation of *B. tuberculosis* into a tuberculous animal, the Koch phenomenon. The intense local reaction which is produced, though essentially of the same kind as in unsensitized animals, comes on more rapidly, is more severe, and usually involves more damage to tissue. The result is, on the whole, beneficial, as it tends to localize and eradicate the bacteria (Zinsser, Ward and Jennings, 1925; Petroff, Branch and Jennings, 1929). Willis (1925<sup>1 & 2</sup>) has shown particularly that *B. tuberculosis* remains localized for a longer time and spreads to the adjacent lymph glands more slowly. This phenomenon has been found to apply to a large number of bacteria or their products and probably operates in many cases of human infection. It has been demonstrated for the *Pneumococcus* by Zinsser and Grinnell (1927), for the *Streptococcus* by Zinsser and Grinnell (1925) and Andrewes, Derick and Swift (1926), for the *Staphylococcus* and *B. typhosus* by Zinsser and Parker (1923). A recent summary of the subject is that of Opie (1929).

### The Bacterial Equipment to Produce Disease, and Bacterial Toxins.

That bacteria differ in their equipment to produce disease is reflected in the common knowledge that the cause of an infectious process is, in a general sense, indicated by the nature and situation of the lesions. Some bacteria seldom invade the tissues beyond the region of their initial entry; the primary lesion may be small or insignificant although the general effects of the infection may be severe, e.g. *B. tetani*, *C. diphtheriae*. Other bacteria characteristically produce a septicaemia. The third and largest group comprises the bacteria associated with a severe reaction which is usually confined to one situation, not necessarily the site of entry; the bacteria may spread by direct extension or by the lymphatics, and may invade the blood. The pathological changes resulting from infection will thus depend on the invasive properties peculiar to different species and on the nature of the injurious substances which they elaborate. The former will determine the distribution of the lesions (the selection of characteristic sites by different species for primary invasion has been mentioned, p. 17), and the latter their character.

The difference between the first two groups mentioned above (*C. diphtheriae* and *B. anthracis*, for example) in respect of their invasive properties has not been explained. It may eventually be found that it is related to the possession of soluble specific substances by the invasive species, but at present it cannot appropriately be termed a difference in virulence. It depends on an equipment for growth in the host peculiar to each species. The spread and distribution of members of the third group is variable. With these (the Gram-positive and Gram-negative cocci, the enteric, *Salmonella* and haemophilic groups, for example), the power to invade may correctly be regarded as an indication of virulence. They may multiply in the blood or be found there because they have been dislodged from foci of disease. Small numbers of bacteria are periodically present

in the blood and may be transferred by it on many undetected occasions. Such is the explanation for the occurrence of osteomyelitis following injury without external lesions, arthritis of gonococcal or other origin, some infections of internal organs arising from chronic foci elsewhere, and other similar conditions.

**Toxins.** The injurious products elaborated by or arising from bacteria fall into somewhat ill-defined groups to which the terms exotoxin, endo toxin, leucocidin, haemolysin, aggressin and split-protein products have been applied. The term exotoxin is often understood to imply that the substance which it denotes is excreted by the living bacterium. Exotoxins have been defined by their properties ; they have not been separated from protein, are non-dialysable, are harmful in small amounts, reproduce the symptoms and pathological changes of the corresponding infection, are not lethal even in comparatively large doses except after a latent period, stimulate the production of antitoxin, can be made non-toxic without the destruction of their antigenic properties. Most of the statements in this definition require revision.

With regard to the elaboration of exotoxins there is no certainty that they are excreted by the living cell. Some toxins are produced abundantly in young rapidly growing cultures (*B. welchii*). On the other hand, *B. tetani*, *C. diphtheriae* and *B. dysenteriae* give off very little toxin in cultures until the phase of rapid growth has ceased, although in the body, toxin may be liberated rapidly. Moloney and Hanna (1921) found that in cultures of *C. diphtheriae* the increase in toxicity took place when the number of viable bacteria was decreasing. Bacterial reproduction and toxin production did not go on at the same time. They obtained no evidence that bacterial autolysis was associated with increase of toxin and did not establish the mechanism by which it was produced. Fildes (1927) observed the liberation of toxin from heated tetanus spores suspended in salt solution. In cultures of *B. dysenteriae* (Shiga) toxin appears in the filtrate some days after the period of growth, at a time when the number of viable bacteria is decreasing and autolysis can be observed (Okell and Blake, 1930). The dried and ground bacteria from 12- to 18-hour cultures contain a potent toxin which appears to be the same as that in the filtrate of older cultures. It does not become detached from the ground bacteria when they are suspended in saline. Dysentery toxin in culture is thus formed during the phase of rapid bacterial growth, but is not liberated until autolysis sets in. It is possible that the rapid production of toxin during infection, by bacteria which form it slowly in cultures, may result from the more rapid disintegration of bacteria in tissues. The difference in the rate at which toxin appears in cultures of various bacteria suggests that the mechanism of toxin-production may not be the same for all species.

Further, as Zinsser (1920) has pointed out, bacteria such as *C. diphtheriae* and *B. tetani* which have low invasive powers can cause serious disease only because they form a potent exotoxin. Species which can invade more

readily could set up severe infection without such potent toxins, and it is possible that those for which exotoxins have not been recognized may form weaker toxins which do not have the marked lethal effect of classical exotoxins, which are not the predominating cause of the lesions resulting from infection, and are not easily demonstrable, although essentially of the same nature. The toxin of *Streptococcus haemolyticus* is an example of one which deviates in some respects from the classical type. It does not affect selected tissues ; in the form of filtrates of cultures it is lethal only in comparatively large doses, and may withstand boiling, yet there seems no doubt that it should be included in the group of exotoxins. Working with concentrated toxin, Hartley (1928) found the lethal dose for rabbits to be as low as 0·2 c.cm. ; its potency was considerably reduced by heating at 56° C. for 1 hour, and it was practically destroyed after 1 hour at 100° C. Further, toxins may kill without an incubation period. *Staphylococcus* toxin (Burnet, 1929) killed rabbits in 2 or 3 minutes in a dose of 0·5 c.cm. given intravenously, and was not always the chief factor in causing death from infection ; nevertheless, an antitoxin can be prepared which neutralizes the toxin in constant proportions. The toxin of *B. cædematis maligni* kills rabbits within 3 to 15 minutes (Vol. III, p. 269).

The difficulty which exists in distinguishing exotoxin from other bacterial poisons, especially with species whose pathogenicity does not depend chiefly upon toxin, lies to some extent in the failure to recognize that all the classical properties of diphtheria toxin, for example, need not necessarily be reproduced, as illustrated in the preceding paragraphs. Exotoxins vary in heat-stability. They may kill without an incubation period, and the lethal dose may be comparatively large. They are not always an excretion from the living cell. They may not reproduce the characteristic lesions of the corresponding infection. The essential criterion is that they should stimulate the production of antitoxin and be neutralized by it ; but antitoxic immunity does not always prevent death from infection by toxigenic bacteria which have invasive properties and are virulent.

The conception of endotoxins as poisons which are held in the cell until it has disintegrated is based on the work of Pfeiffer (1892, 1910) on the toxin of *V. cholerae*, who stated that 'it must be a highly complex and very labile substance which stands in close relationship to the bacterial protoplasm—is perhaps this substance itself'. Bacteria killed by chloroform, thymol or drying were toxic. The injection of suitable amounts of bacteria with a potent antiserum caused the death of guinea-pigs, the interpretation being that the toxins were liberated when the bacteria were lysed by the serum. The principles of this conception have been applied to many species for which exotoxins have not been demonstrated, to account for their injurious effects in the body by assuming that they possess a type of toxin to which the term endotoxin can be applied. The validity of this assumption and its usefulness may well be doubted. To frame a definition for endotoxins as a special group of bacterial poisons is

impossible. They are not antigenic in the sense that antibodies are produced which neutralize their toxic properties to a useful extent. The occasional references in the literature to the presence of anti-endotoxins in immune serum, if there be a warrant for the use of the term in the fact that the serum reduces the toxic effect of infection, have not excluded the possibility that the serum may have contained anti-exotoxin. The presence of exotoxin in the cells of *B. dysenteriae* from young cultures is an example of how it is possible for confusion to arise. The toxin of *B. pestis* (Vol. III, p. 153) has usually been termed endotoxin because it is liberated by disintegration of bacteria in old broth cultures and can be extracted from bacteria grown on agar. It gives rise, however, to antitoxin, a fact which would seem to warrant that it too should be regarded as an exotoxin. It is not certain that the so-called endotoxins are specific or peculiar to each species. Nevertheless, an explanation is required of the mechanism by which bacteria do cause harmful effects apart from the action of exotoxins. The work of Vaughan and his collaborators (Vaughan, 1913, 1917) is important in this connection. They state, 'the cellular protein of all bacteria studied (is) more or less harmful to animals when injected parenterally'. 'The more highly susceptible a given animal is to infection with a given bacterium the more difficult it is to kill that animal with the cellular products of that bacterium. On the other hand, the more highly immune to infection with a given bacterium the more readily does that animal succumb to injections of the cellular products of that bacterium'. One part of the cellular substance of *B. anthracis* to 1,700 parts of body-weight was necessary to kill a guinea-pig, while with *B. coli*, 1 part to 75,000 was sufficient. Guinea-pigs withstood the intra-peritoneal injection of 200 mgm. of cellular proteins of *B. tuberculosis*, but a fraction of a milligram of the protein of *B. prodigiosus* killed them. There was evidence here that the substances of the bacterial cell were harmful in proportion to their susceptibility to be split up *in vivo*. Non-pathogenic bacteria are destroyed in the body more readily than pathogenic species, consequently their cellular substance was more readily disintegrated and was more toxic. Vaughan's view as to the manner in which the cellular substance is acted upon in the body is expressed as follows: 'the body cells supply the ferment, the complex, bacterial, proteins constitute the substrate; the process is essentially destructive, the protein poison is set free'. By chemical methods Vaughan and his colleagues extracted from bacterial and other proteins of diverse origins a poisonous fraction which was very toxic to animals, and his view that the toxic action of bacterial cellular substance results from its being split up in the body is supported by extensive experiments in which many of the features of the reaction to infection could be reproduced by suitable inoculations of protein. It is possible that the facts which arise from his work will not explain fully all the toxic effects of bacteria apart from exotoxins but they indicate one important mechanism by which bacteria exert a harmful action.

The nature of the toxin described by Parker (1919), Zinsser (1920) and Zinsser, Parker and Kuttner (1920) is obscure. They obtained from *B. influenzae* and several other pathogenic and non-pathogenic bacteria a non-specific poison which they termed the 'X' substance. It was present in filtrates of young cultures, and in the filtrate of saline suspensions of the growth from young agar cultures. Rabbits were more susceptible than guinea-pigs. After intravenous injection there was a characteristic incubation period of 1 to 1½ hours; only comparatively large doses (3 to 6 c.cm.) were fatal. Repeated injections caused emaciation and death. There was no evidence that this substance was antigenic. It appeared not to be derived from the substance of the bacterial cell. There was no indication that it was formed in the body. Its importance lies in the necessity to recognize its existence in experimental procedures which may lead to its production.

In considering the nature of the poisons of any species of bacterium it would make for clarity if the terms endotoxin, leucocidin and aggressin were avoided. These terms may have served their purpose but have lost their usefulness; they cannot now be held to indicate fundamental distinctions in the nature of bacterial poisons. The exotoxins, split-protein products and the 'X' substance should be susceptible of differentiation. Apart from these the questions that have to be answered in respect to other harmful bacterial products are: What is the mechanism of their production?; Are they peculiar to the species concerned?; What is their action?; How do they react to physical and chemical agents?; Are they antigenic? Only when complete information has been obtained will it be possible to bring knowledge concerning them into an ordered state.

The terms leucocidin and aggressin do not denote any distinctive class of substance. 'Leucocidin' was first applied by Van de Velde to the action of *Staphylococcus* filtrates on leucocytes, and further observations were made by Neisser and Wechsberg (1901) and others. It denotes one form of poisonous action. Similarly the term 'aggressin' denotes an action, and not a special type of substance, and might well be given up. It was introduced by Bail (1905) to explain the results of experiments in which exudates from the peritoneal cavity of guinea-pigs, after the injection of pathogenic bacteria, exhibited antiphagocytic properties, and rendered a sublethal dose of bacteria fatal to normal animals. Such exudates contain a mixture of products of bacterial growth and disintegration, and although the experiments of Bail are not in question the use of a special term to denote a hypothetical substance seems now to be unwarranted. Critical summaries of the subject have been made by Eastwood (1923) and Topley and Wilson (1929).

Bacterial haemolysins may be considered separately because of the ease with which they can be demonstrated. They fall into two groups, those which are exotoxins (e.g. haemolysin of *B. tetani*, *B. welchii*, *staphylococcus*), and those for which no antitoxin can be demonstrated. With

respect to exotoxins it may be difficult to decide whether the haemolytic action is due to the same substance which causes the characteristic tissue injury or whether it is a separate fraction. Haemolysin of both types occurs. The haemolysins for which there is no antitoxin may also cause a reduction in the number of erythrocytes during infection and may be harmful to leucocytes or other tissues, but it is difficult to assess their part in the general effects of bacterial poisoning because it is difficult to obtain them free from other bacterial products. The production of this type of haemolysin is likely to be a function of the rate of bacterial growth, and to adopt the amount of haemolysin formed in cultures as an index of bacterial virulence or toxicity may be merely to make use of a convenient indicator.

## REFERENCES.

ANDREWES, C. H., DERICK, C. L. & SWIFT, H. F., 1926, *J. Exp. Med.*, **44**, 35.  
 ARKWRIGHT, J. A., 1929, *Lancet*, Lond., ii, 963.  
 ARKWRIGHT, J. A. & ZILVA, S. S., 1924, *J. Path. Bact.*, **27**, 346.  
 AVERY, O. T., CHICKERING, H. T., COLE, R. & DOCHEZ, A. R., 1917, *Monog. Rockefeller Inst. Med. Res.*, No. 7.  
 AVERY, O. T. & HEIDELBERGER, M., 1925, *J. Exp. Med.*, **42**, 367.  
 AVERY, O. T., HEIDELBERGER, M. & GOEBEL, W. F., 1925, *J. Exp. Med.*, **42**, 709.  
 BAIL, O., 1905, *Arch. Hyg.*, Berl., **52**, 272.  
 BARNARD, J. E. & MORGAN, H. DE R., 1903, *Brit. Med. J.*, ii, 1269.  
 BAYNE-JONES, S. & VAN DER LINGEN, S., 1923, *Johns Hopkins Hosp. Bull.*, **34**, 11.  
 BROWNING, C. H. & RUSS, S., 1919, *Proc. Roy. Soc. B.*, **90**, 33.  
 BURGE, W. E. & NEILL, A. J., 1915, *Amer. J. Physiol.*, **38**, 399.  
 BURNET, F. M., 1929, *J. Path. Bact.*, **32**, 717.  
 DAWSON, M. H., 1930<sup>1</sup>, *J. Exp. Med.*, **51**, 99; 1930<sup>2</sup>, *ibid.*, **51**, 123.  
 DICKSON, E. C., BURKE, G. S., BECK, D. & JOHNSTON, J., 1925, *J. Infect. Dis.*, **36**, 472.  
 DOWNIE, A. W., 1930, *J. Path. Bact.*, **33**, 563.  
 DUDLEY, S. F., 1929, *Proc. R. Soc. Med.*, **22**, 569.  
 EASTWOOD, A., 1923, *Rep. Publ. Hlth. Med. Subj.*, No. 22, Lond.  
 FILDES, P., 1927, *Brit. J. Exp. Path.*, **8**, 387; 1929, *ibid.*, **10**, 151.  
 FLEMING, A. & MACLEAN, I. H., 1930, *Brit. J. Exp. Path.*, **11**, 127.  
 FORD, W. W. & ELIOT, C. P., 1928, *J. Exp. Med.*, **48**, 475.  
 GRIFFITH, F., 1928, *J. Hyg., Camb.*, **27**, 113.  
 HARTLEY, P., 1928, *Brit. J. Exp. Path.*, **9**, 259.  
 JOCHIMSEN, E., 1928, *Z. Hyg. InfektKr.*, **109**, 96.  
 JULIANELLE, L. A., 1926, *J. Exp. Med.*, **44**, 683.  
 KOHLISCH, 1908, *Z. Hyg. InfektKr.*, **60**, 508.  
 LANDSTEINER, K., 1928, *J. Immunol.*, **15**, 589.  
 LANGE, B., 1926, *Z. Hyg. InfektKr.*, **106**, 1.  
 LANGE, B. & JOCHIMSEN, E., 1927, *Z. Hyg. InfektKr.*, **108**, 66.  
 LEDINGHAM, J. C. G. & ARKWRIGHT, A., 1912, *The Carrier Problem in Infectious Diseases*, Edward Arnold, London.  
 MAGOON, C. A., 1926, *J. Bact.*, **11**, 253.  
 MARTOS, G. Z., 1927, *Z. Hyg. InfektKr.*, **107**, 477.  
 MARX, E., 1927, *Z. Hyg. InfektKr.*, **107**, 472.  
 MCLEOD, J. W., 1915, *J. Path. Bact.*, **19**, 392.  
 MEDICAL RESEARCH COMMITTEE 1916, *Sp. Rep. Ser. Med. Res. Comm.*, No. 2; 1917, *ibid.*, No. 3.  
 MEDICAL RESEARCH COUNCIL, 1923, *Diphtheria*, H.M. Stationery Office, London.  
 MOLONEY, P. J. & HANNA, L., 1921, *Proc. Soc. Exp. Biol.*, N.Y., **19**, 24.  
 NEISSER, M. & WECHSBERG, F., 1901, *Z. Hyg. InfektKr.*, **36**, 299.  
 NEUFELD, F. & LEVINTHAL, W., 1928, *Z. ImmunForsch.*, **55**, 324.  
 OKELL, C. C. & BLAKE, A. V., 1930, *J. Path. Bact.*, **33**, 57.

OPIE, E. L., 1929, *J. Immunol.*, **17**, 329.  
 PARKER, J. T., 1919, *J. Immunol.*, **4**, 331.  
 PÉTERFI, T. & WÁMOSCHER, L., 1926, *Z. Hyg. InfektKr.*, **106**, 191.  
 PETROFF, S. A., BRANCH, A. & JENNINGS, F. B., JR., 1929, *J. Immunol.*, **16**, 233.  
 PFEIFFER, R., 1892, *Z. Hyg. InfektKr.*, **11**, 393; 1911, *Jber. Ergebn. ImmunForsch.*, 1910, Abt. I, **6**, 13.  
 PULVERTAFT, R. J. V., 1929, *Lancet*, Lond., ii, 924.  
 REIMANN, H. A., 1929, *J. Exp. Med.*, **49**, 237.  
 ROSENOW, E. C., 1928, *The Newer Knowledge of Bacteriology and Immunity*, ed. Jordan and Falk, University Press, Chicago, p. 576.  
 ROUS, P., 1925<sup>1</sup>, *J. Exp. Med.*, **41**, 379; 1925<sup>2</sup>, *ibid.*, **41**, 399; 1925<sup>3</sup>, *ibid.*, **41**, 451; 1925<sup>4</sup>, *ibid.*, **41**, 739.  
 SCHÜTZE, H. & ZILVA, S. S., 1927, *J. Hyg., Camb.*, **26**, 204.  
 SWANN, M. B. R., 1924, *J. Path. Bact.*, **27**, 130.  
 TOPLEY, W. W. C., 1926, *Lancet*, Lond., i, 477, 531, 645.  
 TOPLEY, W. W. C. & WILSON, G. S., 1929, *The Principles of Bacteriology and Immunity*, Arnold, London.  
 VAUGHAN, V. C., 1913, *Protein Split Products in relation to Immunity and Disease*, Lea and Febiger, New York; 1917, *Poisonous Proteins*, C. V. Mosby, St. Louis.  
 VOEGTLIN, C., JOHNSON, J. M. & DYER, H. A., 1924, *J. Pharmacol.*, **24**, 305.  
 WÁMOSCHER, L., 1926, *Z. Hyg. InfektKr.*, **106**, 421.  
 WASSERMANN, A., 1895, *Z. Hyg. InfektKr.*, **19**, 408.  
 WILLIS, H. S., 1925<sup>1</sup>, *Amer. Rev. Tuberc.*, **11**, 427; 1925<sup>2</sup>, *ibid.*, **11**, 439.  
 ZINSSER, H., 1920, *J. Immunol.*, **5**, 265.  
 ZINSSER, H. & GRINNELL, F. B., 1925, *J. Immunol.*, **10**, 725; 1927, *J. Bact.*, **14**, 301.  
 ZINSSER, H. & PARKER, J. T., 1923, *J. Exp. Med.*, **37**, 275.  
 ZINSSER, H., PARKER, J. T. & KUTTNER, A., 1920, *Proc. Soc. Exp. Biol.*, N.Y., **18**, 49.  
 ZINSSER, H., WARD, H. K. & JENNINGS, F. B., JR., 1925, *J. Immunol.*, **10**, 719.

### **Virulence and Antigenic Complexity of Bacteria in relation to Epidemic Disease.**

BY W. M. SCOTT.

The prevalence of bacterial infections causing disease is determined by four factors, (1) the natural susceptibility of individuals in the affected population; (2) the immunity they acquire as the result of infection (whether this latter has produced recognizable disease or not); (3) the current dose of infection, which is a resultant of two factors, (*a*) the frequency of opportunity for transmission, and (*b*) the actual mass of infective material, and (4) the virulence of the infecting bacteria.

Factors (1) and (2) are discussed in Chapters II and III, in so far as they determine infection in the individual case. Their application in explaining the spread and persistence of disease in a community is the special concern of epidemiologists. Factor (3), the dose, is extremely difficult to measure experimentally, under conditions in any way approaching those found in nature, though its importance is well recognized, both in determining the severity of the single case (cf. tuberculosis) and the general prevalence (cf. the association of a high incidence of enteric fever with a low level of sanitation). The data provided by Glover (1918) for cerebrospinal fever provide, perhaps, the nearest approach to precision in correlating dose with epidemicity; his 'danger-line' carrier rate of

20 per cent. and his 2½ foot bed-spacing, which diminishes the number of carriers and cases, are numerical quanta governing the dosage necessary for the maintenance of this disease in epidemic form.

For factor (4), the virulence of the infecting bacteria, abundant data are available to prove that strains of the same bacterial species may show large variations in their capacity to multiply in the body and cause death when inoculated into experimental animals. But no process of rise and fall in this respect among the infecting bacteria has been shown to occur in association with the rise and fall of epidemics or epizootics. Even for the differences in malignancy of different outbreaks of the same disease, there is little direct proof that variations in virulence are the cause. One must remember, of course, the difficulty that, in some of the most important epidemic diseases, virulence tests with the causative bacteria on experimental animals may have little bearing on the behaviour of the same bacteria in man, e.g. in the enteric group of infections; but even in outbreaks of infective disease in animals there are no data correlating grades of virulence with rise and extinction of the epizootic wave. Some inductive epidemiologists, indeed, see no necessity for including variation in virulence of the infecting agent among the factors involved in the rise and fall of epidemics (cf. Kermack and McKendrick, 1927), and even experimental epidemiology (cf. Webster, 1923, 1924, and Webster and Burn, 1927) has not detected any sufficient variation in this respect. It is true that Topley and his collaborators (1928) have found that the severity of different epizootics can be correlated with grades of virulence (*B. aertrycke* in mice), but they justly point out that this does not establish rise and fall of virulence as an important factor affecting the form of the epizootic wave itself. The missing link in the proof is a demonstration that strains of high virulence are relatively frequent during a rise and that strains of low virulence predominate during a fall in the incidence of the disease.

Nevertheless, the hypothesis that variations in virulence of bacteria, i.e. in their infective power, are, at least in part, the cause of fluctuations in epidemic prevalence is so obvious and direct in its application that, if experimental epidemiology has failed to find facts supporting it, the fault probably lies in the experimental methods. What support can be obtained for it from data more indirect, but, perhaps, cumulative in their effect? For this purpose two sets of observations are applicable, those dealing with the 'rough' modification of infective bacteria and those recording variations in antigenic composition in bacterial species during epidemic and inter-epidemic periods.

#### *The 'Rough' Modification.*

The nature of 'rough' modification and the conditions under which it occurs in the various species have been fully dealt with by Arkwright, its discoverer, in Vol. I. The points of importance here are its association with complete or partial loss of virulence and the fact that it can be

produced by growth of the normal virulent bacteria in 'immune' serum. It is true that we cannot yet be certain that the latter phenomenon occurs also *in vivo*, i.e. that the normal process of recovery from a bacterial infection is the replacement of the virulent strain inhabiting the animal by its rough, non-virulent modification ; nor is the 'rough' change known to occur in all pathogenic species. Further, there are few observations to show that 'rough' modifications become the common form of the specific bacterium to be found during the decline of an epidemic or epizootic. But the implications of the facts already known as to 'roughness' and virulence are so obvious and so far-reaching that it is right to assume its influence on the course of epidemics and to make special efforts in future work to collect the missing data.

#### *The Diversity of 'Type' Characters.*

The diversity of antigens which confers 'type' characters on strains within a bacterial species has been the subject of many observations during the past twenty years. Primarily, of course, this diversity in serological characters is of importance in the practical identification of such species. Strains of meningococci, *Salmonellas*, dysentery bacilli or pneumococci cannot be identified precisely by their reactions to an antiserum prepared against a single strain ; to such an antiserum some strains may fail to react at all, or may react only on application of much higher concentrations than those necessary for the homologous culture. Further, it is presumed by analogy, and is, in part, proved that the prophylactic and therapeutic activity of antibacterial sera for these species is also dependent on exact adaptation of the serum to the antigenic complex of the infecting strain ; for details see the chapters treating of the various bacteria in this System. In other species, e.g. the diphtheria bacilli, the influenza bacilli, the haemolytic streptococci, &c., though serological type characters have not hitherto been extensively employed in identification or in therapeutic application, the same diversity of type is encountered.

#### *The Antigenic Spectrum.*

But there emerges, from the mass of work thus undertaken for practical considerations, a body of facts of great theoretical interest. It has become plain that there is in each species a sort of antigenic spectrum, with groups of strains, the 'types', arranged in bands which, in some cases, are sharply marked off from neighbouring bands, but, in other cases, shade into each other owing to the discovery of strains possessing characters common to two or more 'types'. In some species (e.g. *Meningococci*, *Pneumococci*, scarlatinal *Streptococci* and dysentery bacilli of the Flexner species) the 'types' of specially common occurrence are limited in number ; a majority of the strains currently encountered in disease belong to three or four 'types', while, outside these, which we may conveniently call, without prejudice, epidemic types, there are numerous, but not unlimited, 'types' which are much less common and are often

lumped together in practical work as a 'scrap-heap', e.g. Group IV *Pneumococci*, 'untyped' dysentery bacilli, &c. In the *Salmonella* species epidemic types are well-marked, e.g. *B. typhosus*, *B. paratyphosus A* and *B*, *B. enteritidis*, *B. aertrycke*, *B. suis*, while others, equally definite antigenically, are of much less common occurrence. In this species, in contrast with others, the 'types' have been founded on heat-labile components (flagellar antigen) which seem to play a relatively minor part in the virulence and immunity-behaviour of the species; but the heat-stable components of *Salmonellas* can also be used to form types as with other bacteria. In some species, e.g. diphtheria and influenza bacilli, the 'types', though varying in the frequency with which they are encountered, do not show this segregation into an 'epidemic' and a 'scrap-heap' group (cf. Vol. II, p. 369).

The connection between virulence (infective power), and antigenic composition is, thus, established, (1) by the loss of virulence in the 'rough' modification, and (2) by deduction from the fact itself of a special incidence of the 'types' in disease. The question is, now, what is the reason for the existence of the 'spectrum' of antigens within these pathogenic species. Has it any relation to the distribution of the respective bacteria in apparent health and in actual disease? Has it been necessary for each of these bacterial species, in order to maintain itself, to provide a variety of 'types' so as to circumvent a general immunity in the subjects it frequents—and a consequent hostile environment—acquired towards an undifferentiated or archaic form of the species? Does the 'scrap-heap' correspond to a preliminary and tentative evolution in this respect and the 'epidemic' type to a more successful solution of the difficulty by the bacterium, but a solution subject to failure and retrogression to the 'scrap-heap' phase of existence, or even to a limbo of still less differentiated forms (cf. the Hofmann bacillus in relation to *C. diphtheriae*)?

These questions are easy to raise but hard to answer, and a complete discussion of the pros and cons for each bacterial species is quite beyond the province of this System. But enough has been said to indicate that variations in virulence and antigenic complexity of the bacterium are concerned in the genesis and probably also in the extinction of epidemics and that the study of their relation to these is one of the most important tasks for the experimental epidemiologist.

#### REFERENCES.

GLOVER, J. A., 1918, *J. Hyg., Camb.*, **17**, 350, 367.  
 KERMACK, W. O. & MCKENDRICK, A. G., 1927, *Proc. Roy. Soc. A*, **115**, 700.  
 TOPLEY, W. W. C., GREENWOOD, M., WILSON, J. & NEWBOLD, E. M., 1928, *J. Hyg., Camb.*, **27**, 396.  
 WEBSTER, L. T., 1923, *J. Exp. Med.*, **37**, 231, 781; **38**, 33, 45; 1924, *Amer. J. Hyg.*, **4**, 134.  
 WEBSTER, L. T. & BURN, C., 1927, *J. Exp. Med.*, **46**, 887.

## CHAPTER II. NATURAL IMMUNITY.

By J. C. G. LEDINGHAM (LISTER INSTITUTE, LONDON).

### **Introduction.**

NATURAL immunity, in the widest sense, may be defined as the sum total of those forces which the animal body can oppose to noxious influences of whatever kind that may disturb its ordinary economy. For the purposes of the present chapter it may be defined in less teleological terms as the sum of those constitutional factors which researches of modern times in pathology, bacteriology, immunology, and biochemistry, have shown to be chiefly concerned in the interaction between the tissues of the host and any environmental harmful influence of bacterial origin. As we shall be concerned in this chapter almost solely with constitutional factors of resistance to bacteria and their products, it is important to make clear at the outset that these factors are at any time the expression of an inherited equipment modified by favourable or unfavourable environmental influences. A strict line of demarcation between such natural immunity and the specific active immunity that is reached by recovery from a particular disease or by deliberate immunization is not always possible, inasmuch as we have reason to believe that to some extent at least, active immunization of specific type by certain bacteria or their products may be going on without the usual manifestations of infection by such specific organisms. It is usual to speak of such immunity as being 'naturally acquired' in opposition to an otherwise similar order of active immunity following deliberate immunization. We do not know to what extent 'naturally acquired' specific immunity may be due to the influence of an '*infection inapparente*' or a repetition of such, but recent epidemiological researches have at least produced a considerable amount of evidence in support of that origin.

In the following pages an account is given of such defence mechanisms as are capable of exact study and of methods by which such defences may be enhanced or depressed. It is not proposed to discuss the more general influences such as those of climate, race, age and sex, &c. The available information on these matters is better sought in existing epidemiological and statistical treatises, though, doubtless, as experimental epidemiology increases its scope, we are likely to secure more exact information on these less readily appraised factors.

**The Basic Defence Mechanisms residing in Body Tissues and Fluids.***Introductory.*

The subject matter of this chapter will comprise an account, necessarily brief, of the various antibodies and other substances concerned in defence that can be demonstrated in body fluids under normal circumstances by well-recognized laboratory methods, and of various other defence mechanisms, particularly those manifested by mucous surfaces as part of their resistance to saprophytic invasion. Natural resistance in the widest sense is not to be regarded as a function of this or that antibody or defence process. It is a function of many variables, some of which are definitely recognizable and capable of quantitative titration, while others, probably of equal importance, have so far evaded precise scientific treatment. Some of these latter variables, particularly those concerned with local immunity processes and the functional activity of the reticulo-endothelial system, are treated separately under definite headings for the very obvious reason that the phenomena with which they deal have been studied, advisedly or inadvisedly, in rather watertight compartments; but it must be recognized that through all the various mechanisms of defence there runs a common thread which links them into a complete whole. It would be tempting to treat the development of these defence mechanisms in the course of evolution. It was, indeed, Metchnikoff's zoological training which gave him the insight to investigate in higher animals the significance of certain phagocytic activities noted by him in *Daphnia*. The study of phagocytosis, perhaps the most important defence mechanism in the body, then had its birth. We are not, however, sufficiently informed with regard to immunological processes in the lower orders of vertebrates and invertebrates to be in a position to pursue with confidence the comparative method of treatment.

The subject of antibodies generally and their demonstration is treated elsewhere (Chapter VI), and doubtless some inevitable overlapping must take place between the matter of this and other chapters, but here it is proposed to deal with normal antibodies and mechanisms as far as possible from a functional standpoint and in so far as they illuminate problems of natural resistance.

*Inflammation.*

In the course of an address on natural immunity (Ledingham, 1922) the writer explained that analysis of the works of the great masters of pathological anatomy showed how the phenomena of inflammation came gradually to be regarded in the light of defence mechanisms. Marchand's inaugural address as Professor of Pathology in Leipzig in 1900 had for its title 'Die natürlichen Schutzmittel des Organismus', and here he sought to describe in terms of defence the many and various cell changes in the inception, course and repair of inflammatory processes. The advent of bacteriology and its offspring immunology was, indeed, largely

responsible for these newer teleological conceptions. That they have materially stimulated advance in many spheres of pathology, few would deny, however much they regret the departure from the strict mechanistic outlook that these conceptions involve. The criticism offered by certain writers that such conceptions as 'defence', 'reaction', 'invasion', 'battle between host and parasite' and the like are invalid, is based on the ground that the cell, which apparently leads a passive existence in normal health, is made to behave in disease as if it had a personal conflict to wage with the invader and in the waging of this conflict cut all the links which formerly bound it to the body-complex. We say, for example, that a cell of the reticulo-endothelial system reacts to blockade by phagocytizing the dye that happens to enter its environment, or that a dose of antigen stimulates a certain cell to produce antibody. In terms of mechanism we should have to be able to explain why a dye granule which has reached, perhaps by an intricate route, the immediate neighbourhood of a particular type of cell, finds itself promptly phagocytized by it or left alone, or how it happens that absorption of antigen, possibly in some soluble form, by an appropriate cell is followed by elaboration of antibody. Of these two instances, phagocytosis has been studied fairly intensively in recent years as a physico-chemical process and the not so very ancient view that the leucocyte goes out to seek its prey has been almost entirely abandoned. Of the mechanism of antibody formation by the cell we know next to nothing, though we have got so far as to be able to study the matter *in vitro* in tissue culture—a method which some would recognize as highly artificial.

It will be necessary for some considerable time yet to employ these despised teleological terms which express apparently 'purposeful' reactions. Knowledge of a function subserved by a cell is useful, though we may be ignorant of the precise mechanism by which the function is exercised. Researches on 'local' immunity have shown that to a considerable degree purely local cells and tissues, e.g. of the dermis, respond to the insult of viruses, &c., as if they had little or no connection with the body-complex, and further, that states of immunity can be detected in such limited areas without any very strong echo in the immunity of the body-complex. There is some justification, therefore, for the temporary use at least of teleological terms and in this chapter free use will be made of them. It is not to be assumed, however, that the mechanistic aspect is being ignored.

#### *Bactericidal Elements in Blood.*

It has been usual to date the discovery of bactericidal elements in shed blood from the work in this field of Nuttall (1888), who showed clearly by experiment *in vitro* that blood had the property of destroying organisms of the *anthrax-subtilis* group seeded therein—which property was not to be ascribed, as Metchnikoff then taught, solely to the action of phagocytosis by living cells.

I would, however, trace the development of this important defence mechanism of the body, viz. humoral defence, to Lord Lister's experiments of 1880-81 on the keeping quality of ox-blood when removed aseptically from the animal. He showed that such blood had no inherent tendency to 'putrefaction' and, moreover, that it could be seeded with small amounts of tap-water or even considerable amounts of dust without the setting up of detectable growth. What struck Lister in this experiment was the contrast with what he had found to take place in a substance like milk which showed profuse growth when a single bacterium was introduced. Linked with this experimental demonstration by Lister of the presence of bactericidal or growth-inhibiting elements in serum was his teaching that 'it is only in a state of unhealthiness that the ordinary forms of bacteria can enter the circulation and establish themselves in the organism'. I add two further quotations to show that Lister was fully cognisant, not only of purely humoral bactericidal agents in serum, but also of phagocytosis by living cells, to which subject Metchnikoff's early work had directed his attention.

'I happened to be the first to draw attention to the antiseptic agency of living structures and there is perhaps no one who attaches greater importance to it than I do. Without it surgery in former days would have been absolutely impossible'. 'These two great truths, then, have been taught us by advancing science: that normal serum is not a good soil for the development of attenuated microbes and that bacteria introduced among the tissues, if in not too concentrated a form, are disposed of by phagocytosis. The result is that microbes in the form in which they are present in the air are unable to develop in our wounds: and thus we are able to disregard in our experiments the once dreaded atmospheric dust'.

Buchner (1889) extended Nuttall's work with whole blood to serum and showed that serum entirely free from leucocytes was capable of killing certain organisms. The heating of serum at 55° C. for 30 minutes destroyed this property. The next step was to show that the bactericidal action exerted by this thermo-labile body in normal serum, to which Buchner gave the name of alexin, depended on the presence of two factors, a thermo-stable normal amboceptor and a thermo-labile alexin or complement in the Ehrlich terminology.

This combined action has not been so clearly established in the case of normal bactericidal serum as in specific bactericidal or haemolytic serum. A discussion of the available data would not be suitable here, but reference may be made to papers by Moxter (1899) and Wechsberg (1902). What is important to emphasize here is the heat-labile character of the alexin in Buchner's sense and the necessity of distinguishing it from certain heat-stable bodies present in normal sera and known as the  $\beta$ -lysins (see below). It would appear that while certain types of micro-organism (e.g. of the typhoid-cholera-dysentery groups) are readily susceptible to the action of alexin, others are more susceptible to the

action of the thermo-stable bodies or  $\beta$ -lysins (e.g. *anthrax-subtilis* group). Also, while the action of alexin can be increased by specific immunization, there is no evidence that immunization with an organism capable of being acted upon by  $\beta$ -lysin increases the amount of  $\beta$ -lysin in the serum.

Before passing to the discussion of the opsonins, tropins and  $\beta$ -lysins of normal sera, it may be convenient to refer to some recent studies dealing with bactericidal effects produced by 'whole' blood.

#### *Recent studies on bactericidal action of 'whole' blood.*

In these more recent studies an attempt has been made to investigate the capacity of 'whole' blood—generally defibrinated blood—to effect *in vitro* the destruction of organisms which are not believed to be susceptible to the bactericidal or bacteriolytic action of plasma or serum. The organisms which come under this category and which have been chiefly studied are *Staphylococcus*, *Streptococcus*, *Pneumococcus*, *B. pestis*, &c. The technical methods employed have developed along somewhat different lines in this country and in America, but the general principle has been to retain defibrinated blood in contact with a definite quota of test organisms and thereafter to estimate by microscopic, plating or other methods the number of surviving organisms. Wright, Colebrook and Storer (1923) have elaborated for this type of investigation a method by which the test materials are contained in small sealed cells which permit the direct counting of such survivors as have developed, *in situ*, into visible colonies. It is doubtful whether the information gained by these *in-vitro* procedures with whole blood accurately reflects what takes place when organisms are introduced into the blood-stream and their fate followed by methods based on Wysskowitsch's fundamental work. It was at first believed that the destruction of organisms as tested by survival counts was due not to intracellular digestion in phagocytes but to the action of bactericidal substances liberated from the bodies of leucocytes. It is now, however, agreed that phagocytosis, as might have been expected, plays the predominant part and, indeed, it has been shown by Fleming (1926) that when defibrinated blood is freed of leucocytes by filtration through cotton-wool the bactericidal power of such blood for pyogenic cocci is reduced to nil. These recent methods, therefore, involve simply an extension of the earlier opsonic techniques sufficient to permit the testing of final destruction of the phagocytized test organisms by the intracellular ferments. Whether the technique as practised can give full information on this important point is doubtful, as the possibility of the occurrence of inhibitory or bacteriostatic effects cannot be rigidly excluded. Nevertheless, these methods have helped us to understand the potentialities for actual destruction residing in phagocytes which have taken up test organisms sensitized by the available opsonin.

Some applications of the method to problems of natural immunity to specific infections are noted in the course of this chapter. Here it may be mentioned that it has been found possible by Malone, Avari and

Naidu (1925) to correlate the well-known differences in susceptibility to *B. pestis* of Bombay and Madras rats, with differences in the bactericidal potency of whole blood towards this organism.

*Opsonins and Tropins of Normal Serum.*

Wright and Douglas (1903) were the first to draw attention to the presence in normal serum of a substance which is able to sensitize micro-organisms for phagocytosis by the polynuclear leucocyte. To this substance, which was thermo-labile, they gave the name of opsonin. Some years previously substances possessing similar properties had been detected in certain immune sera, e.g. in antistreptococcal serum (Denys and Leclerf, 1895) and in antipneumococcal serum (Mennes, 1897). The almost complete destruction of this opsonic antibody which took place on heating the serum, undoubtedly gave rise to the suggestion that a single thermo-labile body was at work, but it was soon shown that normal serum like immune serum contained, even after inactivation, a heat-stable residuum which of itself could effect sensitization. In some immune sera in fact it was found that the opsonizing action was little, if at all, reduced by heat. In 1904 Neufeld and Rimpau drew attention to these thermo-stable bacteriotropins in antistreptococcal and antipneumococcal sera, and in 1905 G. Dean independently recorded their presence in anti-staphylococcal, antidysertery and antityphoid sera.

Muir and Martin (1906, 1907), from their work on the combining properties of the opsonins of normal serum and the tropins of immune sera, concluded that these two bodies were essentially different, and Neufeld and Hüne (1907) came to a similar conclusion. G. Dean (1907<sup>1</sup>), on the other hand, demonstrated in a series of ingenious experiments the fact that inactivated normal and immune serum were both capable of having their sensitizing action on micro-organisms greatly enhanced by the addition of small quantities of fresh serum (complement). Though he fully admitted that the tropins of immune sera could act alone, probably by virtue of 'heightened specific properties', he was persuaded that complement played an essential part in the process. In normal serum the amboceptor is present only in small amount, hence the great destructive effect of heat on normal opsonin. The demonstration of normal amboceptor has in fact always presented difficulties which have not even now been satisfactorily surmounted. The writer (Ledingham, 1907) attempted to secure evidence of the presence of normal opsonic amboceptor by digesting inactivated normal serum with tubercle bacilli and adding the digest after centrifugalization to a fresh opsonic system. The result was invariably an inhibition of this system, which the writer attributed to fixation of the complement in the fresh system by the free sensitized antigen present in the digest. The inhibition was non-specific, and, indeed, it has long been recognized that the opsonin of normal serum is of non-specific type and can be completely absorbed by digestion with any bacterial suspension.

In recent work by Gordon and Wormall (1928) and Dunlop (1928) an attack on this problem of the constitution of natural antibody has been made by fresh methods based largely on the knowledge acquired in recent years of the so-called complement-components or fractions into which complement can be split. This subject is dealt with elsewhere (Chapter X) and reference to it is made here simply because the experimental results obtained by these authors support the view that normal serum contains an amboceptor of relatively non-specific type capable of acting as a sensitizer in conjunction with complement. Recent advances also in our knowledge of the constitution of bacterial antigen (H and O antigen) and of the corresponding anti-H and anti-O constituents of immune serum which also differ in heat-resistance (Felix and Olitzki, 1929) may yield fresh information on the constitution of the normal and immune opsonins and their relation to the tropins. The corresponding antibodies to these antigenic elements so far studied have been agglutinins, bactericidins and complement-fixing substances, and there would appear to be some evidence (Braun and Nodake, 1924) that it is the heat-stable antigen which gives rise to effective opsonin in serum and that the normal antibody is of the anti-O type. With this question of antigen and corresponding antibody is linked up that of bacterial virulence and the doctrine of chemotaxis. The fact that virulent strains are much less easily phagocytized (if at all) than non-virulent varieties has long been recognized, and the former are also known to be much less susceptible to intracellular digestion. The discharge of negative chemotactic bodies by virulent organisms causing a repulsion of leucocytes in their vicinity was long ago postulated in explanation of this phenomenon. Evidence for the existence of such bodies has not definitely been produced and it would seem more probable that difference in the constitution of the antigen is the deciding factor. Marchand (1898), for example, showed that virulent streptococci could be washed and boiled without their 'leucocyte-repelling' function being abolished.

#### *Leukins and $\beta$ -Lysins.*

Leukins are heat-stable bactericidal substances present in extracts of leucocytes, while  $\beta$ -lysins are somewhat less heat-stable bactericidal substances present in normal serum. The study of the leukins was one outcome of the struggle between the contending views of Metchnikoff and of the rising school of humoralists. Alexin, according to Metchnikoff, was essentially a product of cell deterioration in shed blood, which was thus handed over to the serum. His view, now quite untenable, was that complement did not circulate as such in plasma. Attempts were therefore made to determine whether bactericidal substances were capable of demonstration in leucocytic extracts prepared in various ways (alternate freezing and thawing, extraction by saline or by saline and inactive serum), and, if so, whether they betrayed the physical and functional properties of alexin. Work on this subject has been undertaken only by a very

limited number of authors, of whom Hahn (1895), Schattenfroh (1897, 1899), Weil (1911), Pettersson (1906, 1908<sup>1 & 2</sup>), Schneider (1909) and Petrie (1904) may be mentioned, and the great majority of the papers relevant to this subject appeared towards the end of the first decade of this century. Since then Pettersson (1928<sup>1 & 2</sup>, 1929) alone appears to have continued the study of these bactericidal bodies, particularly in relation to the heat-stable bodies in serum known as  $\beta$ -lysins, which possess very similar properties. The bactericidal action of leucocytic extracts has been tested against a variety of bacterial species, and it is notable that some species are highly susceptible while others are not. Also, while the leukins of one species of animal appear to have a definite bactericidal action on organisms of the typhoid-colic group, those of another species may have no such action, but may prove active against organisms of the *anthrax-subtilis* group. Though the quantitative data on which the evidence is based are in some cases faulty for various reasons (lack of controls, variations in duplicate plate-counts, temporary growth inhibitions, &c.), there is no doubt that these extracts have as a rule a very definite bactericidal action, especially on organisms of the saprophytic spore-bearing aerobic group, and that in this respect they present a close affinity to the anthracidal bodies of egg-white, to the  $\beta$ -lysins and possibly to lysozyme. The leukins are inactivated by half an hour's heating at 75° C. (Pettersson, loc. cit.). It is claimed that inactivated extracts can be reactivated by a minute amount of the unheated extract, but this complexity of structure has not been generally accepted. A point of more importance is the relation of the leukins to the  $\beta$ -lysins of normal serum. The term  $\beta$ -lysin was given to these bodies to distinguish them from the leukins on the one hand and from the alexins or  $\alpha$ -lysins, which depend on complement, on the other. Like the alexins, the  $\beta$ -lysins are, according to Pettersson, of complex constitution. The portion analogous to immune-body is denoted the 'activable substance' and that corresponding to complement the 'activating substance', which is destroyed at temperatures ranging from 63 to 70° C. Neither removal of salt by dialysis nor shaking with ether destroys the activating substance, and it can also be recovered in the active state from acetone precipitates if the precipitation is carried out with cooled materials. Activation of inactivated serum is effected by adding a small volume of native serum or of mid-piece obtained by the CO<sub>2</sub> method. Platelet and leucocytic extracts are also, according to Pettersson, capable of acting as activators, and in this connection may be recalled the importance attached by Gruber and Futaki (1907) and by Barreau (1909) to the platelet extracts or plakins as factors in natural immunity to anthrax. Unlike amboceptor, the  $\beta$ -lysins in inactivated serum do not fix the sensitive bacteria. For binding, the activating substance must be present. Also unlike amboceptor, the  $\beta$ -lysin-content of the serum is not increased by immunization by a sensitive organism. Among the organisms susceptible to the action of alexin are the typhoid-colon group, *V. cholerae*, *B. pyocyaneus*, *Pneumococcus*

and *Streptococcus*, while those susceptible to  $\beta$ -lysin include *B. subtilis*, *B. anihracis* and the *B. proteus* group. It is not possible at present to assess the sphere of action of  $\beta$ -lysin in natural defence. Its energetic action on the *anthrax-subtilis* group has suggested that it may play an important role in natural and acquired immunity to anthrax—a notoriously difficult problem in immunology. It is quite clear, however, that a wide field is open for further research into the constitution and interrelationships of  $\beta$ -lysins, leukins and lysozyme, whose properties and fields of action appear to have much in common.

#### *The Presence in Normal Mammalian Sera of Complement-fixing Bodies and Agglutinins for Various Bacteria.*

In discussing the nature and properties of normal opsonins, tropins,  $\beta$ -lysins and leukins reference has been made to questions of specificity and resistance to heat. Investigation of the behaviour of normal antibodies in these respects deserves further attention from the light it might throw on the development and properties of antibodies produced in response to immunization. A discussion of this subject so far as it concerns normal complement-fixing bodies and normal agglutinins for bacteria will be found in papers by Mackie and Finkelstein (1930) and Gibson (1930). The former authors show that the complement-fixing bodies in normal sera possess some degree of specificity for the microbe with which they are found to interact. Gibson also demonstrated a considerable degree of specificity of the normal antibacterial agglutinin. With regard to heat-resistance, the complement-fixing bodies possessed the heat-lability of complement while the agglutinins were much less susceptible to heat, being generally inactivated at temperatures between 60 and 65°C. Another point of interest was the fact that the complement-fixing bodies were fully developed in the serum of young animals, whereas the normal agglutinins for bacteria, like the normal haemolysins and the normal haemagglutinins, were in defect.

#### *Functional Role of the Antibacterial Agglutinins.*

The functional significance in body defence of the presence of agglutinins in the sera of animals, whether normal or specifically immunized, has in the past received scanty attention as compared with that of other antibodies, such as lysins, opsonins and tropins. There can be little doubt, however, that in the disposal of organisms which gain entrance into the blood-stream or are deposited in the tissues, the presence of agglutinin in the blood or lymph facilitates very materially, not only their further treatment by lytic substances, if such are also present, but also their elaboration by phagocytes in the circulating blood and in the reticulo-endothelial system. Experimental evidence of the occurrence of agglutination *in vivo* and the favouring influence of such on the phagocytic processes in the circulating blood and tissues is amply afforded in papers by Bull (1915), and for a full discussion of the matter the reader may be referred to a special review of the subject by Bailey (1928).

*Phagocytosis.*

The fundamental importance of phagocytosis in natural defence necessitates some brief account here of the phenomena associated with that process, more particularly with reference to recent studies of these phenomena by physical and physico-chemical methods.

*The phagocytizing cells.* In test-tube experiment, with bacteria or formed cell elements such as red cells as the phagocytizable objects, the polynuclear cell is the phagocyte *par excellence*. In the body, on the other hand, as Metchnikoff pointed out, the mononuclear cell (macrophage) of pleural, peritoneal, and dermal exudates plays a most important role as a phagocyte, not only of formed elements such as effete red cells, effete polynuclears or inanimate material (dyes, &c.), but also of bacteria. The mononuclear cell comes into action, as a rule, following the preliminary interaction between the bacteria and the polynuclear cells. Elsewhere (p. 66) is discussed the role of the mononuclear cells in the blood and reticulo-endothelial system.

*Importance of sensitization by serum of the object exposed to phagocytosis.* That bacteria are more readily phagocytized after sensitization by serum scarcely requires mention, and the statement applies particularly to organisms of low virulence. There is an inverse relationship between degree of virulence of the organism and its liability to be phagocytized even in the presence of serum. Particles of inanimate material (carbon, starch, &c.) and saprophytic bacteria are capable of being phagocytized in the absence of sensitizing serum, but it is notorious that sensitization greatly increases the intake even of inanimate particles. The writer (Ledingham, 1909) showed, further, that animals immunized with melanin yielded a serum in the presence of which melanin granules were more abundantly phagocytized than in the presence of normal serum.

*Nature of the surface of the particle exposed to phagocytosis.* The nature of the surface is of importance. Fenn (1921<sup>1 & 2</sup>) for example, has shown that in comparative experiments with carbon and quartz particles, while the chances of contact with the phagocytes were the same, the chance of phagocytosis of a carbon particle was twelve times that for a quartz particle. When the experiment is carried out in an acid medium at pH 6·0 the quartz is more readily phagocytized than the carbon, and the suggestion is that a change of charge on the particle has taken place. On the other hand, when the particles are suspended in gum, the carbon granules are, according to Fenn, taken up more readily than the quartz whatever the acidity. Earlier experiments on spontaneous phagocytosis by Schütze (1914) demonstrated the increased phagocytosis of cocci which took place in the presence of acid. He pointed out that in neutral and alkaline suspensions cocci and leucocytes possess a negative charge, whereas in acid suspensions containing haemoglobin or other amphoteric electrolyte both cocci and leucocytes take on a positive charge and migrate to the cathode. The suggestion was that the increased phagocytosis

in the more acid medium was the result of diminished charge on both leucocyte and bacterium and consequent lowering of the repulsive forces.

*Stages in the phagocytic process.* The writer (Ledingham, 1908) showed that at low temperatures sensitized cocci stick to the periphery of leucocytes without entering them. Temperature differences such as that between 18 and 37° C. did not affect the intake provided that the organisms presented to the leucocytes were previously sensitized. It thus appeared that increased amœboid energy following rise of temperature was not necessarily associated with increased intake. It has, in fact, been shown that phagocytosis goes on perfectly satisfactorily in tubes in which the phagocytes are being constantly rotated and consequently prevented from spreading on the glass surfaces. With regard to the final stage of phagocytosis or inclusion and the relation of this process to surface tension changes and electric surface charges on leucocyte and phagocytable object, the reader must be referred to reviews of these problems by Fenn (1928), Ponder (1926) and Mudd *et al.* (1929). For lack of space this highly interesting problem cannot be effectively discussed here. Reference, however, may be made to the writer's (Ledingham, 1912) mathematical analysis of the phagocytic intakes under conditions in which saline suspensions of phagocytes and washed sensitized bacteria were incubated together. The process of inclusion progressed numerically as an adsorption process.

*Changes in functional activity of leucocytes in health and disease.* For information on this subject the work of Glynn and Cox (1909) and Hektoen (1911) should be consulted. Experiments designed to detect differences in functional activity demand careful planning to ensure controlled conditions, but there would appear to be definite evidence that such changes do occur both in health and disease, and that the functional activity of the leucocyte cannot be regarded as a constant quantity.

*Influence of temperature.* The temperature-coefficient of phagocytosis *in vitro* of bacteria and of quartz and carbon particles has been studied by Madsen and Wulff (1916), Madsen and Watabiki (1919) and Fenn (1922<sup>1 & 2</sup>). Analysis of Madsen and Watabiki's figures (phagocytosis of bacteria) by Fenn gave a temperature-coefficient of 2.0 over the range 0 to 35° C. Fenn's own figures for phagocytosis of quartz and carbon revealed no constant coefficient throughout the range 20 to 37° C. Within the range 23 to 27° C. the coefficient worked out on the average at  $18 \pm 6$ , while within the range 30 to 37° C. it fell to  $1.41 \pm 0.06$ . Fenn suggests that the cell substance at the lower temperatures is too rigid and that a small increase of temperature by raising the fluidity of the cell protoplasm rapidly increases the rate of phagocytosis, thus yielding a high temperature-coefficient.

An interesting question is the influence of body temperature on phagocytosis by leucocytes removed from the blood of normal healthy persons or cases of fever. Madsen and Wulff (1916) show that in normal

persons and animals the maximal phagocytosis by the corresponding leucocytes is obtained at the temperature of the body. (Man, 37° C.; guinea-pig, 39° C.; hen, 41° C.). If such leucocytes are exposed (*in vitro*) to slightly higher or slightly lower temperatures, the intake falls. In febrile human cases, on the other hand, the maximal phagocytosis by the corresponding leucocytes is obtained usually at the febrile temperature prevailing at the moment of removal of the leucocytes. The elevated temperature, therefore, does not affect this defence mechanism. In poikilothermic animals (e.g. frogs) there is no such optimum. At all temperatures examined the leucocytes of the frog exhibited the same degree of phagocytic action.

#### *Lysozyme (Fleming).*

An interesting development in our knowledge of natural defence mechanisms was the demonstration by Fleming (1922) of a ferment-like bactericidal substance, denoted by him lysozyme, present in various secretions and tissues of the body and more especially in the tears and cartilage, where an action on the test organism can be detected in a concentration of 1 in 40,000 and 1 in 1,300 respectively. Lysozyme does not dissolve all species of organisms. Its action is best seen when tested against a certain saprophytic micrococcus isolated by Fleming and called by him *M. lysodeikticus*. On this coccus the dissolving action of lysozyme is very rapid when the latter is used in high concentration. Cocci exposed to it in a broth-tube at a temperature of 45 to 50° C. go to pieces in a few moments and the tube becomes clear. Another method of demonstrating its action is to place here and there on a thickly grown plate of *M. lysodeikticus* drops of tears, or egg-white or saline, when it will be found that after a minute the coccal growth becomes clear under the tears and egg-white, but remains unchanged under the saline. Though lysozyme is particularly concentrated in the lachrymal secretion it is found, according to Fleming, in all secretions except urine, sweat and cerebro-spinal fluid, and in all tissues, being, as we have said, in greatest concentration in cartilage. The behaviour of lysozyme on *M. lysodeikticus* is precisely similar to that of egg-white, which, as Laschtschenko (1909) and Rettger and Sperry (1912) showed, was capable of destroying saprophytic organisms of the *anthrax-subtilis* type with great rapidity. Apart from animal fluids and tissues, lysozyme was capable of demonstration in certain garden vegetables though in much weaker concentration.

In addition to *M. lysodeikticus*, enterococci were specially susceptible to lysozyme, whereas organisms of the typhoid-colic group were not affected.

*Physical properties of lysozyme.* Protein precipitants, trichloracetic acid, alcohol and acetone bring down lysozyme, and it is said to have been prepared in a protein-free state by Wolff (1927). It is destroyed, as a rule, in half an hour at a temperature of 75° C., though in tears complete destruction is not obtained except by a few minutes' boiling. It does not

pass through a porcelain filter except in the later stages of filtration when large amounts of lysozyme-containing fluid are dealt with, the first portions to come through being devoid of lysozyme. (Compare the behaviour of complement on filtration, see Chapter X.) In a salt-content of 0·5 per cent. lysis was most rapid, while at 5 per cent. concentration it was abolished.

*Development of resistant strains.* As in phage experiments the occurrence of resistant strains was noted in areas where inhibition had occurred. These strains retain the property for many months of repeated subculture. Resistance to egg-white could also be induced, and Fleming notes that such resistant strains became insensitive also to the bactericidal action of 'whole' blood and to intracellular digestion by human leucocytes. Fleming has also recorded evidence showing that the lytic power of a lysozyme is not removed by digestion with the test organism, but, indeed, shows some degree of increase, its behaviour in this respect recalling that of bacteriophage. There can be little doubt that the presence of this powerful ferment in the secretions bathing the surfaces of eye, nose and mouth must have a profound influence in preventing any prolonged settlement of saprophytic invaders.

The properties of lysozyme, so far as they have been studied by Fleming and his collaborators, undoubtedly show a close general resemblance to those of the leukins and the  $\beta$ -lysins. The bactericidal substance in all three shows considerable heat-stability : their action is confined largely to certain types of saprophytic bacteria, and is usually exceedingly rapid : and there is no evidence that the leukin-content of leucocytic extracts or the lysozyme-content of secretions and tissues can be increased by active immunization : Fleming has shown that leucocytes contain abundant lysozyme as tested by his methods, and he regards the bactericidal substance discovered many years ago in egg-white as essentially lysozyme, by his tests (see Fleming, and Fleming and Allison, 1922, 1924, 1926, 1927, 1929).

It would seem, therefore, highly probable that Fleming's lysozyme may connote or comprise a whole series of bactericidal substances, whose presence has been reported in various secretions and tissues of the body. The light which Fleming and his collaborators have been able to throw on this question has been doubtless facilitated by the happy choice of a highly susceptible test organism. The choice would, in fact, correspond to that of *B. subtilis* for the testing of egg-white, leukin, and  $\beta$ -lysin. It is suggested by Fleming that the proteolytic ferment (microcytase) which is believed to be responsible for the intracellular digestion of bacteria is of similar nature to lysozyme on the ground that strains rendered resistant to lysozyme also become resistant to intracellular digestion. It seems more probable, however, that the lysozyme-resistant strains have really become more serum-fast and consequently less susceptible to intracellular lysis—a well-recognized phenomenon. In view of the comprehensive sphere of action of lysozyme and its ease of demonstration

it seems almost unnecessary to refer to descriptions of bactericidal substances detected by other methods in tissues and mucosæ. Future research must decide to what extent it is valid to group all these bodies in one category. Some sort of distinction based on the nature of the bacterial group susceptible to the particular tissue-bactericidin would appear possible. Löwenberg (1928), for example, finds that duodenal juice from healthy persons is strongly bactericidal for typhoid and dysentery organisms, and suggests that the acidity of the gastric juice, coupled with the bactericidal action of the duodenal mucous membrane, render the entrance of *B. typhosus* into the body via the tonsils more probable.

Levinthal (1929) also attempted to determine whether normal mucosæ possessed an inhibitory action on diphtheria bacilli and pneumococci. Rabbits and guinea-pigs were bled out and excised pieces of tracheal, vesical and intestinal mucosæ were inoculated *in vitro* with these organisms. Pneumococci did not survive either on fresh or cooked mucosæ, while *C. diphtheriae* was killed out on fresh mucosæ but grew well on cooked. Fleming's methods for demonstration of lysozyme might possibly have given more convincing results, but in any case it is doubtful how far lysozyme action can extend to organisms of the more parasitic type.

#### **Induced Modifications of Natural Resistance by Parenteral Injection of Proteins, Colloids, &c., and by Various Physical and Chemical Agents.**

In this section is discussed what may be regarded as the better documented evidence extracted from a vast literature bearing more or less directly on the question of loss or enhancement of natural defence, artificially induced, the term 'natural defence' being employed to cover all those mechanisms which may be presumed to enter into the sum-total of the resistance factor.

##### *The Influence of Parenteral Injection of 'Non-Specific' Substances.*

This subject, which has now reached vast dimensions and has many ramifications, takes its origin from the classical experiments of Issaeff and Pfeiffer and Issaeff (1894), who showed that the intraperitoneal injection of substances like normal broth, peptone, urine, &c., so altered the local defences of the guinea-pig's peritoneum that the animal was able to withstand doses of living cholera vibrios that would otherwise have been lethal. The precise mechanism of this enhanced defence is not yet fully known. A local leucocytosis certainly occurs, and possibly in the aftermath of this leucocytosis, primarily of polynuclear type though later of the macrophage order, the injected test-dose of vibrios may be more rapidly dealt with either by direct phagocytosis or more probably by the known bactericidal elements present in extracts of broken-down leucocytes. In the light, however, of the more recently developed conceptions which have gathered round the varied phenomena of what is

called 'protein shock', it is not unlikely that the local cellular antibody-forming elements of the reticulo-endothelial system respond to the injection of the non-specific substance by increased proliferation and consequently by a greater mobilization of normal antibody. The local tissue is, in fact, rendered more responsive and more alert, while the constituent antibody-forming cells become more permeable to foreign protein, with the result that the otherwise fatal dose of vibrios meets with host elements better situated to deal with the foreign vibrio. There is no evidence that resources of this kind are unlimited and consequently capable of dealing with organisms highly virulent for the host. Where, however, an animal has the power to deal successfully with small numbers of an organism which, only in larger doses and introduced at a particular site, is capable of producing a lethal effect, there is no doubt that the sum total of the local and general defences can be definitely enhanced by these artificial stimuli. The local inflammatory reaction induced by the non-specific stimulus conduces to the localization of the battle between host and parasite—an arrangement of definite value to the former—and it is almost certain that the phenomenon as a whole is to be placed in the category of those other phenomena in which an inflammatory reaction induced at one spot serves as the main locus of interaction between host and parasite when the latter has been introduced elsewhere, e.g. by the intravenous route.

Though the credit belongs to Pfeiffer and Issaeff of demonstrating the influence of purely non-specific substances in enhancing local defence, the still earlier experiments of Klein (1893) have a very important bearing on later developments. Klein, in the course of an attempt to show that Haffkine's anticholera vaccine did not possess the specificity attributed to it, inoculated guinea-pigs intraperitoneally and even subcutaneously with various organisms, *B. coli*, *B. prodigiosus*, *B. proteus*, &c., and found that these animals, like others previously inoculated with cholera vibrios, were refractory to otherwise lethal doses of this organism introduced into the peritoneum. Accordingly, on the strength of these experiments, he denied the specificity of Haffkine's preventive method. What he really proved, however, was the fact that a form of resistance of a temporary character could be produced by the injection of a heterologous bacterial protein. In a sense Pfeiffer and Issaeff's work may be regarded as an extension of Klein's demonstration to still less 'specific' interferences. Nor can we ignore observations such as those of Treves (1894) on the factors of success or failure attending operations on the peritoneum. He states, for example, that in many of the successful operative procedures for perforations and the like, there had long been antecedent visceral or peritoneal trouble.

Since the early work of Klein and of Pfeiffer and Issaeff remarkably few experimental data on similar lines have been recorded, most probably because it has not been thought necessary to include in lethal dose tests on specifically immunized animals, control series immunized with

heterologous bacterial protein or other non-specific material. Nevertheless, further knowledge is highly desirable, particularly as to the degree and duration of such resistance as may be conferred by non-specific stimulants. The work of Gay and his collaborators (1920, 1921, 1923<sup>1 & 2</sup>, 1924) on the protective action of macrophage exudates in streptococcal infections of the rabbit's pleura, and that of Nakahara (1925) on the increased resistance of the peritoneum to experimental infection, conferred by similar exudates appearing in response to the injection of oil, deserve particular mention as they emphasize Metchnikoff's claims with regard to the major importance of these cells in defence, and at the same time bring the whole matter within the purview of the reticulo-endothelial system. The recent employment of substances such as tapioca as local stimulants to antitoxin production led Sicard, Paraf and Robert-Wallich (1927) to test its capacity to enhance defence when introduced into the guinea-pig's peritoneum along with a lethal dose of vibrios. Survival was the rule in such circumstances. If the tapioca was given 16 hours before the intraperitoneal dose of vibrios the animals died, though more slowly than the controls. Given subcutaneously, this substance had no influence on the response to intraperitoneal injection of cholera vibrios.

In concluding this introductory section dealing with basic evidence, it should be stated that success, in securing by non-specific stimuli the desired temporary enhancement of resistance, is often capricious, probably because of our ignorance of the sequence of events in individual cases and of the times most suitable for introducing the test-dose with some certainty of obtaining its neutralization. The individual factor must be of enormous importance. A similar caveat applies to many of the experimental data in connection with the parenteral injection of 'non-specific' proteins and colloids and the antibody response thereto. To this subject we now come.

#### *Nuclein, Deuteroalbumose, Starch, &c.*

Ledingham and Bulloch (1906), Bedson (1915) and others have shown that the parenteral injection of nuclein or autolysed bacterial protein is followed in a remarkably short time (30 minutes to 2 hours) by a demonstrable material increase in normal opsonin, which lasts perhaps 24 hours. The rise in opsonin-content is accompanied by the leucocytosis which has long been known to succeed the injection of substances like nuclein. That leucocytosis *per se*, however, is not a deciding factor is shown by the fact that the blood-serum obtained from animals after the injection of much more powerful leucotactic substances such as tallianine shows no demonstrable increase of opsonin (Ledingham and Bulloch). Analysis of the mechanism of this increased opsonic effect by Bedson did not reveal any accompanying increase of thermo-stable opsonin or tropin. It is probable that the increased titre is due not to a new development of antibody—which is hardly likely in the brief time—but to increased

mobilization of antibody in the blood-stream following the stimulation of the normal antibody-forming tissues by the nuclein. The phenomenon in all probability forms the basis of those remarkably early accessions to circulating antibody following the injection of readily assimilable bacterial autolysates and is almost certainly of a non-specific character.

An increase in the bactericidal action of 'whole' blood has also been obtained by Fleming (1928) with nuclein and by Prausnitz and Meissner (1925) with substances such as yatren and aolan. In these cases the increase is almost certainly due in the main to an increased opsonic effect of short duration. How far increase in the total number of leucocytes produced by the nuclein contributes to the enhanced bactericidal effect of whole blood is doubtful. Fleming (1928) states that the new leucocytes which appear in response to nuclein are efficient as phagocytes and that consequently their increase will be beneficial in the total effect. Ledingham and Bulloch, on the other hand, placed on record a large series of experiments which certainly suggested that leucocytes appearing in response to tallianine have greatly reduced phagocytic efficiency.

We are not primarily concerned here with antibodies specifically produced, but reference cannot be avoided to precisely similar effects following the injection of non-specific substances in specifically immunized animals. Since Cole (1904) first demonstrated the influence of a small dose of homologous antigen on an animal which had once been immunized but whose antibody-content had been allowed to fall to a low level, the question of the secondary stimulus and its mechanism has become of great theoretical and practical importance (see Chapter IV). Dreyer and Walker (1909) showed that the injection of a heterologous bacterial protein was capable of inducing an increase of a specific agglutinin-titre, but it has also been demonstrated (Tsukahara, 1921; Jaggi, 1923, *et al.*) that the parenteral injection of nuclein or deuteroalbumose may also produce an apparently new development of specific agglutinin. Jaggi noted that the new antibody curve following the injection of the non-specific agent did not behave like the true anamnestic curve, which rises rapidly to its maximum, but ran like the primary curve, exhibiting the usual rise to the maximum in nine or ten days.

In recent years considerable attention has been given to the problem of influencing the normal antibody-content by parenteral injection of metallic salts and organic and inorganic colloids. The results obtained have been on the whole somewhat discrepant. They have never possessed the certainty exhibited by specific antigens parenterally injected. Only in a proportion of trials does the expected result take place. Though the metallic salts and colloids are uncertain in their action on normal antibody when injected by themselves, there would appear to be some definite evidence that in conjunction with specific antigen they are capable of enhancing very materially the development of specific antibody (Davesne, 1927; Steabben, 1925, 1926, *et al.*).

*Ultraviolet Rays.*

According to Bessemans and Seldeslachts (1928<sup>1 & 2</sup>) and Bessemans and Nélis (1928), who review this question, the appearance of typhoid agglutinins in rabbits is accelerated and their maximum titre increased by ultraviolet irradiation, but only if such irradiation is performed several times before the injection of antigen, the idea being that the stimulus of irradiation mobilizes the immunogenic forces of the body. Curiously enough, haemolysin development was not similarly influenced. Colebrook, Eidenow and Hill (1924) have demonstrated a temporary increase of the bactericidal power of whole blood following exposure of the animal to ultraviolet irradiation. A too long exposure apparently causes a fall of titre. With regard to the rather variable data on the influence of sunlight and ultraviolet irradiation on the resisting power of rachitic rats to a specific experimental infection, a communication by Robertson (1929) may be consulted. Both in animals and in man, opinion as to the precise influence of these radiations has not yet quite crystallized. According to Taylor and Murphy (1917) and Mottram and Kingsbury (1924) X-rays render mice and rats more susceptible to tuberculous infection. No significant effect, however, was noted by Browning, Gulbransen and Russ (1919) with the X-ray doses they employed. On the other hand, ultraviolet radiations caused some inhibition of the omental tuberculous infection in Schütze and Zilva's (1927) experiments with rats. The latter radiations usually produced a definite rise in the leucocyte count.

*Fatigue.*

The data on this question are reviewed by Bailey (1929), who noted the development of rapid and severe systemic infection in rabbits infected intranasally with Type I *Pneumococci* and exposed to fatigue. Boycott and Price-Jones (1926) also found that while fatigue did not influence the mortality of rats after intraperitoneal inoculation of *B. enteritidis* Gaertner, it very greatly increased the subsequent illness and mortality following oral administration of this organism. According to Bailey, fatigue favours agglutinin production. In exercised animals the rate of formation of agglutinin increases and a higher maximum is reached than in control animals. The explanation of this increase is probably to be sought in an increased permeability of reactive tissues to antigen (see reticulo-endothelial system, p. 66).

*Conditioned Reflexes.*

The claim has been made by Metalnikov and Chorine (1926), Polettini (1929) and others that reflexes of the kind studied by Pavlow may have an importance in immunity reactions and defence. Polettini, for example, immunized rabbits with killed *B. typhosus* and before each injection of antigen he 'submitted' the animals to the sound of a trumpet or motor horn. At the close of immunization to this accompaniment of sound, the agglutinin titres were estimated. Subsequently, on submitting some of the animals to sound alone, their agglutinin titres rose. It is too early to evaluate the significance, if any, of such experiments.

**Nutritional Factors in relation to Problems of Natural Immunity.**

It has been a commonplace of lay and medical thought from time immemorial that normal well-being and its maintenance are in some obscure way dependent on the amount and composition of the diet, but it is only within the last fifteen years, which have witnessed a determined attack on nutritional problems by the experimental method, that our knowledge of such dependence has acquired a more precise character. That deprivation of a particular diet element might lead to a particular and clinically well-defined disease-syndrome was not only recognized, at least in the case of scurvy, by scientific voyagers like Captain Cook, but was also empirically, yet successfully combated. The recognition also of certain debilitated states and liabilities to intercurrent infectious disease that have invariably followed in the wake of famine and starvation need only be mentioned. The influence of famine, for example, on the susceptibility of the organism to intestinal infections was brought forcibly to the writer's personal notice in connection with the appalling mortality, among starved Armenian and Assyrian refugees in Mesopotamia, from bacillary dysentery and enteric disease. Here, however, I propose to deal solely with data which have emerged from the experimental investigation of nutritional defects under thoroughly controlled conditions and which have a bearing on one or other of the many problems grouped under the wide concept of natural resistance. The general object of such experiments has been to enquire whether the metabolic changes consequent on diet disorders are reflected in, or accompanied by, changes in the defence mechanisms capable of being measured *in vitro* or *in vivo*. At least four important problems have been submitted to experiment: (1) the influence of nutritional defects on normal antibody-content; (2) the capacity of the organism subjected to nutritional defect to manufacture antibodies in response to parenteral injection of antigen; (3) the liability of the experimental animal to intercurrent infective disease; and (4) the susceptibility of the animal to an experimentally induced infection during the period of dietetic deficiency or avitaminosis.

Among the first to investigate immunity problems in connection with induced nutritional disturbance was Zilva (1919), who studied antibody-production and complement-content in animals exposed to various deficiencies under controlled conditions. Series of rats were placed on diets deficient in the following respects: (A) low content of mineral constituents, iron, calcium, potassium, sodium, chlorine and phosphorus; (B) diets deficient in certain amino-acids by employing, as the source of protein, low-level caseinogen or gliadin; and (C) diets deficient in each of the vitamins, A, B and C, i.e. in AB, BC and AC combinations. These various diets produced, as a rule, restricted growth of the animals exposed to them. The controls were placed on unrestricted diets. No significant difference between experimental and control animals was noted in the

magnitude of the antibody response (agglutinins and complement-fixing bodies) to injections of *B. typhosus* antigen, and complement-content was not affected. As Zilva pointed out, these negative results could not be taken to imply that normal resistance was unaffected by the diet defect. Smith and Wason (1923) tested complement-content and the normal bactericidin-, tropin- and opsonin-content of the blood of rats on a rickets-producing diet. The one interesting fact that emerged from their study was the diminished bactericidal titre of the rachitic rat-sera. As in Zilva's experiments complement was not affected. Antibody production has also been studied by Werkman (1923) on rats, rabbits and pigeons on diets deficient in vitamins A and B in view of alleged diminution of resistance to experimental infection under these conditions (see below). No significant departures from the normal could be detected in any of the manifestations of the antibody-producing mechanism. Werkman, however, suggested that phagocytosis in the living animal might be detrimentally affected by the fall of normal temperature occurring in vitamin starvation.

With regard to experimental infection during a period of avitaminosis a considerable body of evidence is available though much of it is poorly documented and insufficiently controlled. Werkman made the interesting observation that rats on diets deficient in A readily succumb to anthrax—an infection to which these animals are normally notoriously resistant. Pigeons and rats on diets deficient in B also became susceptible to anthrax. Similarly, guinea-pigs inoculated during the scorbutic stage with pneumococci of human origin were found by Findlay (1923) to be distinctly less resistant than controls on normal diet. The loss of resistance he was inclined to attribute to feeble leucoblastic reaction in the blood-forming organs. On diets deficient in B, pigeons proved also highly susceptible to pneumococci, particularly during the late stages of the induced polyneuritis, when the body temperature was considerably lowered. Antipyretics alone appear to be capable of inducing a state of reduced resistance to pneumococci in pigeons (Strouse, 1909), and here may be recalled the classical experiment of Pasteur and Joubert (1878) on the susceptibility to anthrax of the cooled hen, which they attributed, perhaps with unwarranted simplicity, to the reduction of the normally high body temperature.

With regard to experimental tuberculosis in guinea-pigs, rats and mice under restricted diets, it is not yet possible to interpret precisely in terms of vitamin deficiency the results obtained. Schütze and Zilva, who discuss the available data, found that guinea-pigs on restricted diet but containing vitamin C in adequate amount lived on the average 50 and 87 days after infection with virulent human and attenuated bovine tubercle bacilli respectively, while controls on abundant diet lived on the average 88 and 202 days respectively. There was also some evidence that excess of cod-liver oil inhibited the formation of tuberculous omental masses in intraperitoneally inoculated rats.

Webster and Pritchett (1924) compared the mortality resulting from infection *per os* with *B. typhi murium* of mice on a bread and milk diet with that from similar infections in mice kept on McCollum diet. The latter series exhibited the greater resistance and these proved also more resistant to the toxin of *B. botulinus*. The two types of diet used by Webster and Pritchett were not such as to produce obvious disease manifestations and the experiments illustrate simply what differences in resistance are liable to be encountered when large experimental series are distinguished by slight differences only, in the generosity of the diet. Much emphasis is laid by Pritchett (1925<sup>1&2</sup>) on the fact that her diets were not distinguished by presence or absence of a particular vitamin or vitamins. The addition to her basic diet of 5 per cent. of an animal fat yielded an increased resistance to infection *per os* with *B. typhi murium*. In the experiments of Schmidt-Weyland and Költzsch (1928) scorbutic animals proved more susceptible to experimental infection with pneumococci and pasteurella organisms administered *per os* and by spray.

Perhaps the most interesting experiments are those in which note has been taken of infections occurring spontaneously in the course of experimentally induced nutritional defects. Such experiments would naturally afford evidence of alterations in natural resistance to infection by organisms which, being saprophytic, would not in normal circumstances be liable to invade the body. In the more recent of these experiments, an attempt has been made to attribute any loss of normal resistance to the absence of a particular vitamin from the diet. Clear-cut deductions of this kind are perhaps somewhat premature, as we have little precise knowledge of metabolic changes under different defective diets. Doubtless, as observations under strictly controlled conditions accumulate, our knowledge of the precise resistance-diminishing factor in the test diet will become more ample. In Schmidt-Weyland and Költzsch's work the deaths of guinea-pigs on scorbutic diets were found to be due to pneumococcal or pasteurellar pneumonic lesions according as the season favoured one or other of these two prime infections of laboratory stocks. The observations undoubtedly indicate the presence of a seasonal factor. Consequently, deaths would be attributed primarily to interference with natural defence by the scorbutic state and secondarily and immediately to infection by the particular saprophytic organism that happened to be prevalent on the mucosæ. Green and Mellanby (1928), who review some of the evidence pointing to vitamin A as the 'anti-infective' agent, reported that rats on a diet deficient in A (containing vitamin D) exhibited at death, in a very large percentage of cases, abscesses in the tongue, ear, nasal sinuses and kidney. Bladder calculi first observed by Osborne, Mendel and Ferry (1917), and since by a number of workers, were also found in a few cases. Control animals on a generous basal diet showed, when killed, no evidence of infective processes at all corresponding to those met with in the deficient series. In a more recent paper (Green and Mellanby, 1930), the authors

ascribe high anti-infective powers to carotene. A more precise knowledge of the bacteriology of these intercurrent infections is very desirable in view of the light that might be thrown on the exact field of action of the induced resistance.

In this connection attention may be drawn to certain intercurrent and lethal infections liable to occur among experimental guinea-pigs on diets either deliberately or inadvertently deficient. Spontaneous pneumococcal infections in guinea-pigs may assume severe forms, especially in breeding sows. Epizootics of this kind have been frequently noted since Theobald Smith's early observations in 1913 and they seem to be particularly frequent in animals kept near the scurvy line, as Wambscher (1927) has shown. His view is that the lowered resistance induced by the food deficiency results in an auto-infection by the guinea-pig's own parasitic pneumococci. To pneumococci of other than guinea-pig origin it is notorious that guinea-pigs are resistant. Since the winter of 1924, when acute pneumococcal infections among breeding sows at the Serum Department of the Lister Institute were first observed by Petrie (1930), a study has been made of the factors which appeared to contribute to the maintenance of such epizootics. A more generous diet of green food diminished the incidence of infection and increased the fertility. Though mothers rarely died in the puerperium, occasional deaths among litters from pneumococcal infection continued to occur during the winter months. The suggestion was that some essential factor necessary for the nutrition of the foetus was missing from the mothers' blood during the latter exhausting period of the winter and that the absence of this factor was less harmful to the mothers than to the young. So far it has not been possible to show that radiation of part of the breeding stock with ultra-violet light has materially increased the natural resistance to pneumococcal infections of this character.

#### *Diet and Tissue-reactivity to Various Stimuli.*

It is convenient here to refer to certain interesting phenomena encountered in connection with intradermal or subcutaneous introduction of toxins in animals suffering from nutritional defect. The local inflammatory swelling which is so marked a feature of the response to diphtheria toxin in the guinea-pig was found by Arkwright and Zilva (1924) to be greatly reduced when the experimental animals were maintained on a restricted diet leading to arrest of growth and loss of weight. Both the simply restricted diets and the diets deficient in C affected the local response similarly. While local swelling was minimal, pleural effusion was completely absent. Death, however, from toxæmia was neither retarded nor accelerated. Bieling (1925<sup>1</sup>) made similar observations and noted that the pleurisies and even the adrenal congestions were often in abeyance. The same author (1925<sup>2</sup>, 1926) showed that the immunization of rats and guinea-pigs with tetanus anatoxin led to a diminished production of antitoxin when the animals were underfed.

*Summary.*

While there is little evidence that nutritional defects disturb the antibody-forming mechanism, there is ample proof that resistance both to spontaneous and induced infection is profoundly affected. The precise parts played by particular vitamin deficiencies in producing this state of lowered resistance are not yet clear. Generally restricted diets may act similarly to diets lacking particular vitamins. Seasonal influences in liability to specific laboratory scourges must be duly considered in evaluating the effect of nutritional disturbance on general resistance. The normal excitability of reticulo-endothelial tissue is very greatly reduced by defects of diet which lead to loss of weight.

**Breeding Experiments in relation to Natural Immunity Problems.**

The entry of the geneticist into the field of immunity is of quite recent date, though long overdue. The value of pure line strains for experimental purposes such as the testing of susceptibility to various infections and intoxications is obvious. With regard also to those peculiar resistances that distinguish certain species and races it is possible that the geneticist may be able to throw light on the hereditable factors on which these resistances depend and which may be capable of exploitation to utilitarian ends. A full discussion of the geneticist's aims in this sphere of work is impossible here and reference can be made only to data which concern the subject matter of these chapters.

Pritchett (1925<sup>1 & 2</sup>) has employed five different strains of mice in the course of her investigation of the susceptibility of mice to mouse typhoid. Of each of these strains of mice more than 500 animals were tested in the course of 12 months, with the result that notable and clear-cut differences in susceptibility were detected. She consequently draws attention to the desirability of determining the normal variations in susceptibility of any given strain of mice before deciding what is a normal or abnormal response to an injurious agent. These same five strains of mice exhibited also seasonal variations in their response to mouse typhoid, viz. a high mortality in the spring, a lower death-rate in summer and a subsequent autumn rise in the number of deaths. Evidence that resistance may be a factor capable of hereditary transmission is afforded by the experimental work of Webster (1924), who bred selectively from mice which had withstood an infection *per os* with mouse typhoid. Inbreeding through several generations from these surviving mice resulted in a very definitely increased resistance to infection on the part of the progeny of selected parents. The resistance was to some extent of a non-specific character, as evidenced by the fact that the progeny, like the parents previously tested, possessed an increased resistance to substances such as corrosive sublimate given *per os*. Apparently, therefore, the transmissible resistance-enhancing factor contains both specific and non-specific elements. The converse experiments, viz. to inbreed from susceptible females with production of

a progeny still more susceptible has also given definite results. Mothers were collected at random and marked. When the litters arrived the mothers were tested and their mortality curve ascertained. The progeny of those mothers which proved susceptible in these tests were then inbred, and the resulting progeny were found to possess a greatly increased susceptibility as compared with a group of unselected mice. An instance of reduced general resistance accompanying the inheritance of a recessive Mendelian factor is seen in the breeding experiments carried out (see Moore, 1919; Hyde, 1923) in connection with the race of guinea-pigs whose serum contained no complement. The animals which possessed this recessive factor were extremely difficult to rear and in all probability the recessive factor had a lethal linkage.

*Local Resistance to Toxins, &c., in relation to Blood-Group Inheritance.*

The Mendelian inheritance of the blood groups alluded to elsewhere is now generally accepted. With this hereditary transmission of the blood groups an attempt has been made by certain authors, particularly Hirsfeld (1924) and Hirsfeld and Seydel (1925) to link certain resistances, such as that embodied in Schick-negativity, which in reality connotes the presence of diphtheria antitoxin. Though the evidence presented has been severely challenged by professional geneticists (Snyder, 1927) on the ground that insufficient attention has been paid to the requirements that must be conformed with in order to establish such linkage, it seems to the writer very probable, even on the available evidence, that the relative Schick-negativeness in the first year of life and that which is eventually developed in later adult life may depend to some extent at least on hereditary factors, and may not be fully explained on the basis of a naturally acquired specific immunity. It is of considerable interest, for example, that Heinbecker and Irvine-Jones (1928), in the course of their Schick-testing of Baffin Island Eskimos, found that the children under 12 were invariably Schick-positive, while all the adults tested were Schick-negative. In the blood of three persons antitoxin was demonstrated. According to the authors all the available evidence points to a complete absence of clinical diphtheria among these Eskimos.

The occurrence of variations in normal antibody-content (normal lysins, normal agglutinins) corresponding with the age of the subject has recently been studied by Friedberger, Bock and Fürstenheim (1929), who infer from their data that purely biological forces may be concerned in the well-known Schick age-susceptibility curves. It is most probable, however, that both physiological and 'infective' factors are at work, but the matter deserves further investigation on epidemiological and serological lines. The comparative absence in the blood at birth and in the early weeks of life of antibodies such as isolysins and haemagglutinins and their appearance in demonstrable amount in later life must be considered not only in relation to the possible inheritance of a genetic factor which matures only slowly, but also in relation to the responsiveness at

different ages of the antibody-forming mechanism to specific though unrecognized stimuli. Such reactivity or lack of reactivity may also have a hereditary basis.

*Passive Inheritance of Antibodies.*

Mention only may be made here of the passive transference of antibodies from mother to child through the placental circulation or the milk. Such endowments of immunity are of a purely transitory nature.

**Natural Immunity to Particular Infections or Intoxications met with in certain Animal Species and Races.**

There are numerous instances of such natural immunities in the literature dating from the earliest days of the bacteriological era, and attempts to solve the mechanism of the particular resistance have always possessed a strong fascination for workers. Even at the present day it cannot be said that an adequate explanation of the mechanism involved in any one instance of natural immunity is forthcoming in spite of immunological progress. The geneticist can tell us only the mechanism of the inheritance, it is for the immunologist to explain how the naturally immune animals deals with the organism introduced into its system. It is true that many instances of natural immunity in species or races are badly documented. In others, however, such as the immunity of the rat to diphtheria toxin, the immunity of dog, rat, fowl and frog to anthrax, the immunity of the fowl to *Pneumococcus*, and the immunity of certain species to venoms, &c., the ground has been fairly well covered, and it is possible to review in these typical instances the present state of knowledge.

*The Immunity of the Rat to C. diphtheriae and its Toxin.*

The rat possesses an immunity to *C. diphtheriae* and its toxin of such high order as to be very nearly absolute. It is of such order that it can be made to succumb only by the administration of 1,000 to 4,000 guinea-pig m.l.d's (Cobbett, 1899; Coca, Russell and Baughman, 1921; Ramon, Noureddine and Erber, 1928). To what is this immunity directly due? There is no evidence of the presence of diphtheria antitoxin in rat-blood. Coca, Russell and Baughman gave rats 1,000 guinea-pig m.l.d's intraperitoneally, and 24 hours later, when they had bled the animals to death, they injected the whole yield of rat-serum into guinea-pigs, which died of diphtheria toxæmia. It would appear that the toxin is not immediately bound, owing to the absence of appropriate receptors, but circulates as such for some time. Pettit (1914) came to a similar conclusion from his experiments with the filtered urine of rats inoculated from some hours to three days previously with diphtheria toxin. Such urines were capable of producing a toxæmia in guinea-pigs which was neutralized by diphtheria antitoxin. There are, of course, parallel observations in connection with the fate of tetanus toxin and venoms when inoculated into insusceptible animals. Coca, Russell and Baughman obtained evidence that repeated injection of diphtheria toxin into rats led to the production of antitoxin—a

finding, however, which has not been confirmed by Ramon, Noureddine and Erber. The latter authors immunized rats with diphtheria anatoxin and failed to demonstrate antitoxin in their blood. Moreover, the animals were not rendered immune even to two or three rat-lethal doses. They would liken the condition of the rat that is refractory to immunization to that of the child in the first year of life who resists immunization with diphtheria toxin, but who later loses the refractory state, to become susceptible not only to spontaneous attack of the disease but also to artificial immunization. It may be well to note, however, that mere insensitivity of the skin to diphtheria toxin as here observed in no way connotes an insensitivity of the body as a whole to the micro-organism and its toxin.

Sbarsky (1926) recorded a series of experiments the results of which satisfied him that the red cells of the rat were unable to absorb diphtheria toxin *in vitro*, while those of the susceptible guinea-pig did so. To this difference he attributed the natural immunity of the rat. Serious technical errors, however, appear to have crept into Sbarsky's absorption experiments, as pointed out by Schmidt (1927-8). The latter went over the ground very carefully and found no evidence whatever of absorption of diphtheria toxin by red cells whether of rat or susceptible guinea-pig. The writer (Ledingham, 1926) has put on record his observations of the cellular reaction following the introduction of *C. diphtheriae* into the peritoneum and subcutis of the rat. At a period of 48 hours after intra-peritoneal inoculation the exudate contained polynuclears and large mononuclears (clasmacytocytes) stuffed with bacilli. In the peritoneum of the protected guinea-pig, on the other hand, phagocytosis by mononuclears was not observed. When toxin was injected into the rat's peritoneum the exudate taken at a similar period consisted of mononuclears and polynuclears in the proportion of 10 to 1. If diphtheria bacilli are injected into the subcutis of rats or protected guinea-pigs a localized 'abscess' results containing actively phagocytizing polynuclears, and at a subsequent period the 'abscess' is surrounded and finally absorbed by proliferating reticulo-endothelial tissue. In the unprotected guinea-pig, apart from the polynuclear infiltration, and the vascular congestion and haemorrhages, the important feature is the reaction of the reticulo-endothelial system. The reticulo-endothelial cells, including the capillary endothelium, the adventitial cells of vessel sheaths and the histiocytes lying along the connective tissue fibres, all show evidence of karyorrhexis and pyknosis, the end-result being the transformation of their nuclei to large deeply stained spherical or oval masses. The rat response differs from that of the unprotected guinea-pig by the fact that the local reticulo-endothelial cells suffer no initial injury and promptly respond to the insult by rapid proliferation leading to a localization of the foreign protein.

The evidence presented here would support the view that the rat's immunity depends on a lack of responsiveness of its reticulo-endothelial cells to diphtheria bacilli and their toxin. It behaves precisely like the

protected guinea-pig. Both in rat and protected guinea-pig there is no rapid destruction of the bacilli injected into the subcutis. They retain, indeed, some power to multiply locally and they have been recovered in a virulent form from the local site some weeks after injection. Capacity to localize infection at the earliest stage would appear to be of first importance both in the rat and in the protected guinea-pig. Thereafter the infective agent, bereft of the centripetal path of invasion, is left only with the centrifugal means of escape to the exterior by abscess formation and perforation. With regard to the visceral lesions that may be found in rats which do succumb to a fatal dose of diphtheria toxin Pettit (1914) noted changes in kidneys, liver and spleen which recalled those met with in the susceptible rabbit but of a less severe type. Parenchymatous changes were present in the kidney, the liver cells showed coagulation necrosis, while the spleen presented areas in which the cells were pyknosed and karyorrhectic. Like others who have investigated this subject, Pettit concluded simply that the cells of the rat are refractory to the toxin. It has to be remembered, however, that the route of administration of the toxin may be of importance in view of Roux and Borrel's (1898) demonstration of the susceptibility of the rat to intracerebral injection of toxin.

#### *Natural Resistance of the Pigeon to Pneumococcus Infection.*

We have already referred to this question in connection with the influence on the pigeon's immunity of nutritional defects and of deliberate interference with the temperature-regulating function by means of drugs (Strouse, 1909). As Kyes (1916) remarks, it is very doubtful how far it is legitimate to emphasize individual factors and to attribute the immunity of the pigeon to this and other infections (e.g. anthrax) simply to the possession of a high body temperature. Kyes demonstrated that pneumococci after introduction into the blood of pigeons localize in the liver and spleen, where they are destroyed in the course of phagocytosis by the fixed tissue cells. The Kupffer cells of the liver and splenic haemophages remove the injected pneumococci very rapidly. Certainly a leucocytosis is produced by the injection of pneumococci, but the circulating leucocytes show little or no phagocytosis of these organisms. The pigeon's serum also appeared to be devoid of action on pneumococci *in vitro*.

#### *Natural Resistance to Anthrax.*

The writer discussed (1922) the various conceptions which have been held as to the mechanism by which relatively resistant animals (rat, dog, fowl, frog) deal with *B. anthracis* experimentally introduced, and it was pointed out that no one mechanism, however well established in any particular case studied, could be regarded as affording a satisfactory explanation of the resistant state. A state of resistance which in one animal was attributed to enhanced local phagocytosis by Metchnikoff was by another attributed to humoral action. With regard to the latter, the problem is not rendered more simple by the existence of anthracidal

bodies in the serum, not only of highly resistant animals but also of certain highly susceptible species. In the serum of the rat, for example, there is a strong thermo-stable anthracidal body, but while the resistant dog has little anthracidal substance in its serum, that of the rabbit, a highly susceptible animal, contains a fairly powerful anthracidal substance. Efforts to elucidate the mechanism of natural immunity by a study of the response of the animal to experimental infection while suffering from some serious metabolic interference (exposure to extreme cold or heat, antipyretics, vitamin deficiencies, &c.) do not greatly help us to explain the mechanism by which the resistant animal normally disposes of the test organism. This problem for its complete solution must involve an exact comparison, not only of the behaviour of the test parasite (or saprophyte) in the susceptible and resistant animal but also of the behaviour of the host's tissues in response to the introduction of the parasite. Our present knowledge of the pathogenesis of anthrax infection even in susceptible species is still somewhat fragmentary and we are still insufficiently informed with regard to the precise nature of the active immunity to this organism and more particularly as to its expression in terms of serology.

Reference has already been made to the problem of anthrax in relation to local immunity. In an early critique of Besredka's claims with regard to the exclusive vulnerability of the guinea-pig's skin it was suggested by the writer (Ledingham, 1923-4) that the introduction of the organism into the skin, where local defence processes only very slowly came into action, gave time for the evolution of a fully virulent race of anthrax which would overwhelm the defences of the body. This conception appears to be in consonance with the results of recent work on the function of the anthrax capsule—a much-disputed question hitherto. Combiesco (1928<sup>1 & 2</sup>) shows that encapsulation is not a full expression of virulence. Encapsulated organisms of the first Pasteurian vaccine are, according to Combiesco, readily phagocytized by leucocytes. Unencapsulated bacilli of a truly virulent strain, however, are, after introduction into the skin of a susceptible animal, phagocytized for the most part, but those which escape rapidly become encapsulated and multiply to invade the body, mainly via the blood-stream.

As we have said, the content of anthracidal body in the sera of various species varies greatly (see Pirenne, 1904; Gruber and Futaki, 1907; Barreau, 1909), and there would appear to be no relation between such content and degree of natural resistance to anthrax. We have so far no precise knowledge with regard to the relative contents of plasma and serum in the matter of anthracocidin. Very complicated relationships have been postulated in this respect by Gruber and Futaki and Barreau. Gruber and Futaki stated that the destruction of anthrax bacilli in the subcutis of the hen and dog was extracellular in spite of the fact that in these resistant animals the serum is not anthracidal. They believed, therefore, that the destruction must be due to some anthracidal body or activating body handed over to the lymph by the leucocytes or platelets. The whole

matter of anthrax immunity, both natural and acquired, calls for renewed investigation, particularly on combined histological and serological lines, since these have yielded interesting results in other infections to which a natural resistance is offered. It may be noted that blockade of the reticulo-endothelial system has, in animals naturally resistant to anthrax, yielded little evidence pointing to defence rupture. The old experiment of Bardach (1889), who claimed to have broken the immunity of the dog to anthrax by splenectomy, has not been repeated with success by subsequent workers (see Bieling, 1929).

#### *Natural Resistance to Snake-Venom, Tetanus Toxin, &c.*

Available data concerning resistance among animal species to snake-venoms comprise much that is thoroughly well documented and much that is curious lore. Snakes themselves are usually resistant to their own venom when introduced parenterally or by deliberate bite by their own species. They may not, however, be immune to venom elaborated by other poisonous species. With regard to mammals possessing great natural resistance to snake-venoms, the hedgehog and the mongoose are outstanding examples. According to Phisalix (see Marie Phisalix, *Animaux venimeux et venins*, 1922), for a hedgehog of 645 gm. the m.l.d. is 20 mgm. (or 31 mgm. per kgm. body-weight), whereas for a white rat of 110 gm. the m.l.d. is 0·21 mgm. (or 1·8 mgm. per kgm.). It is stated that the young hedgehog is less resistant to viper-venom than the adult. Calmette (1895) found that the mongoose was eight times more resistant than the rabbit to cobra-venom. We are not well informed with regard to the mechanism of the resistance observed in these species and in certain cold-blooded species. Phisalix and Bertrand (1895) found that the blood-serum of the viper contains small quantities of antivenin, so small, however, that 'all the blood of the viper hardly suffices to protect a single guinea-pig against 2 m.l.d.'. The heated serum of the hedgehog also possesses antitoxic properties, while that of the mongoose contains only very small quantities.

How far it is possible to attribute the observed resistance in these particular cases to the presence of specific properties in the serum is doubtful. Phisalix believes that even in the hedgehog one cannot exclude the factor of acquired tolerance. Such factor could, however, only be likely to be effective in regions where hedgehogs and vipers are together prevalent. If, as seems probable, it is the case that the young animal of the species is much less resistant than the adult, while the latter's resistance is associated with the presence of specific antivenins in its blood, the immunity observed would come into the category of naturally acquired immunity. Kraus (1927), we may note, accepts the presence of neutralizing bodies in the sera of resistant animals as adequately explaining the natural resistance observed. At the same time, further information is desired on this subject, particularly with regard to the resistance at various ages and in various environments.

Reference has been made to the resistance of the rat to diphtheria toxin (p. 55) and the phenomenon has been discussed. Certain cold-blooded species (e.g. lizards) show enormous resistance to tetanus toxin, and it would appear that tetanus toxin injected into these animals circulates as such for some time and is only slowly excreted. The phenomenon is perhaps comparable with that of the so-called '*infection inapparente*' (Nicolle) which has been observed, for example, in the rabbit and rat inoculated with typhus virus (see Vol. VII, Chapter XXXIV). These animals present no symptoms of infection but retain the virus in their bodies for some time and may, indeed, respond to its presence by the elaboration of antibodies (e.g. v. *B. proteus X19*).

### The Problem of Local Immunity.

It is convenient to discuss the data relating to this subject under a special heading, not because the phenomena with which it deals could not readily be classified elsewhere, but because the fresh attack on this problem by Besredka (1919-24) and the promulgation of somewhat novel views founded upon the interpretation placed by him on his experimental data have given rise to considerable controversy, with the result that a bulky literature on the subject is now available. Besredka's basic experiments deal with three types of infection, staphylococcus and streptococcus, anthrax and enteric infection, and I propose to indicate briefly the nature of the experimental procedures that he adopted in each case and the interpretations he placed upon his results.

Guinea-pigs inoculated subcutaneously or intradermally with killed staphylococci or poulticed on the shaved abdominal skin with dressings impregnated with filtered or unfiltered staphylococcus cultures, proved to be highly resistant to a dose of living virulent staphylococci introduced subcutaneously 24 to 48 hours later. The rapidity with which this apparent immunity developed precluded in his opinion any possible formation of antibodies, and, indeed, for Besredka the absence of antibody formation was an essential and cardinal feature of all those procedures in which local immunity, whether of skin or other receptive tissue, was induced with accompanying general immunity of the animal. From certain growth experiments which he conducted with staphylococcus cultures he concluded that broth-culture filtrates contained a substance which inhibited the growth of fresh staphylococcus seedlings. This inhibitory action was specific for staphylococcus, and to the inhibiting substance he gave the name 'antivirus'. It is to this body that he attributes the vaccinating action of his poultices. The immunity is not confined to the skin of the poulticed area. There develops according to him a general solid immunity, and other portions of the cutaneous surface receive their immunity not by the development of antibodies and their distribution through the blood but by some form of superficial lymphatic spread. According to Besredka the antivirus in staphylococcus and other filtrates protects the receptive

histiocytes of the reticulo-endothelial system from the action of the virus. They become, in fact, 'desensitized' and capable of dealing fully with the invading organism. The production is thus avoided of any deleterious substance which might otherwise result from the interplay of bacterium and cell and exercise a leucocyte-repelling function. The phagocytes consequently get full play for their activities.

In connection with anthrax, Besredka's claim was that the skin of the guinea-pig is the only vulnerable portion of the animal's anatomy, and by applying to the skin the attenuated Pasteurian anthrax vaccines he claimed to be able to produce a solid immunity of the animal, and in no other way. Similarly, by immunizing rabbits *per os* with killed dysentery and paratyphoid organisms preferably preceded by a dose of ox-bile, he produced a solid immunity to these organisms when administered by the intravenous route. It need hardly be said that the methods employed by Besredka for inducing local immunity have been widely employed therapeutically and apparently with a considerable amount of success, but with this aspect of the subject we have little concern, as, after all, the employment of bacterial filtrates for purposes of therapeutic immunization is only one of many methods that may conceivably elicit a vaccinating response by virtue of some residual antigenic constituent and quite apart from any hypothetical 'antivirus' content. Here we are concerned only with the experiments so far as they illuminate problems of natural immunity, and there can be no doubt that our knowledge of local immunity processes resident in or capable of being elicited in the skin or elsewhere has been advanced by the many new facts which recent studies, based on Besredka's thesis, have established. Before reviewing some of this recent work, however, I think it necessary to draw attention to the classical paper by Cobbett and Melsome (1896), whose contributions to the subject of local immunity antedated Besredka's work by twenty-five years. These authors concluded that the cessation of growth of streptococcus in broth culture was due to exhaustion of the medium and not to the elaboration of some bactericidal substance. Cutaneous erysipelas completely protects the parts directly affected against subsequent inoculation of erysipelas streptococci. The local immunity is absolute while the general immunity imparted is only partial and inconstant. Both immunities are of short duration. When previous erysipelas sites are reinoculated, an accelerated reaction occurs. Filtered cultures and dead streptococci give similar differences as regards local and general alterations in immunity. Cobbett and Melsome rightly attached the greatest importance to the increased reactive powers possessed by tissues which in the past had been the seat of a specific inflammatory process. It cannot be doubted that the studies of Cobbett and Melsome are of fundamental importance in connection with the study of local immunity.

Since Besredka made these claims with regard to the specific effect of poultices impregnated with staphylococcus filtrates in producing a local immunity to the homologous infecting organism, a succession of workers

have repeated these experiments, and have arrived at somewhat different conclusions. Mallory and Marble (1925) made a series of intracutaneous inoculations of staphylococcal filtrate in place of poultices. The result was that the protected area reacted very feebly to virulent staphylococci in comparison with the unprotected area. Protection was also afforded by uninoculated broth. The difference, in fact, was insignificant. The immunity of the protected area was apparent for at least 15 days, while the actual sites of the previous cutaneous inoculation showed a non-specific immunity for as long as 5 or 6 weeks. [Compare the failure of vaccine virus to proliferate when introduced into an old erysipelas lesion (Ledingham, 1927).]

Miller (1927) also used the intracutaneous method and found that the local increase of immunity lasted only 8 to 10 days and could be brought about both by broth and by foreign serum. If the skin was too strongly irritated no increase of resistance ensued—an important observation. Miller does not regard the immunity as purely local, seeing that some degree of skin resistance was produced by intravenous or intraperitoneal treatment. The experiments of Grumbach (1928, 1929) on the antivirus problem were carried out with streptococcal, staphylococcal and *B. coli* filtrates prepared from various strains. It was found that *B. coli* and staphylococci could grow in streptococcus filtrates, whereas streptococcus and staphylococcus were unable to grow in a *B. coli* filtrate. The differences noted appeared to depend on the nature of the foodstuffs required for the metabolism of the various organisms tested. For the immunity experiments Grumbach employed both streptococcus and staphylococcus. The virulence of the former was raised by passage to such degree that 0·05 to 0·001 c.cm. of an 18 to 24 hours' broth culture seeded with a drop of infective heart-blood killed a guinea-pig of 350 gm. after subcutaneous inoculation. Of the *Staphylococcus aureus*, 0·15 c.cm. of a broth culture given subcutaneously killed a guinea-pig in 48 to 72 hours. In the experiments with poultices or multiple intracutaneous application of filtrates, the test of immunity was the survival of the animal from an otherwise lethal dose. The general result of the experiments was that protection against death was afforded when the test-dose of virulent culture was injected into the immunized area. When the test-dose was given subcutaneously, even three cm. away from the immunized area, no recovery took place. Non-specific filtrates or broth gave no protection against death from the test-dose, though in some cases a retardation of death was noted.

There would seem to be good evidence, therefore, for the view he expresses that non-specific agents are potent only against infections of low virulence. Experiments such as those of Grumbach demonstrate that a filtrate of a highly virulent organism can, when introduced into the dermis, so alter the responsiveness of the local tissue that a later injection of the living virulent organism is completely localized. If heterologous filtrates or broth are incapable of imparting this increased

responsiveness of the cells to specific antigen, it is clear that we must be dealing with specific immunization by virtue of the presence of antigenic elements in the filtrates. Grumbach does not discuss this possibility. The fact that inoculation of the test-dose outside the immunized spot causes death would suggest that the apparently specific immunization led to no general immunity and therefore differed in degree only from that due to a non-specific stimulus. The experiments gave no suggestion of the presence of an antivirus whose action extended to all receptive cells of the body from its site of entrance. Similarly, Barg (1928) employed the intracutaneous inoculation of the filtrates in multiple spots (10 to 12) over the whole abdomen but sometimes confined to a restricted area. The test employed was resistance to a subcutaneous inoculation of the homologous strain, the results being read in terms of the dimensions of the infiltrate or necrotic area. As a rule the resistance was local and confined to the area covered by the intracutaneous inoculations, and Barg, like Miller, noted a diminution of resistance in areas in which many spots had been inoculated in a restricted space. Control guinea-pigs inoculated with broth alone showed local resistance. While areas into which staphylococci had been inoculated after bad shaving showed a diminution of resistance, shaving performed 1 or 2 days before the infecting dose conferred an increase of resistance.

The results so far cited are in essential agreement. There is no question that broth and other substances can yield similar results to filtrates when infections of low virulence are concerned. Schweinburg (1928), commenting on the enthusiastic clinical reports of antivirus action, states that he gave broth to physicians without their knowing it and was met by the complaint that the 'vaccine' did not act, so that possibly for therapeutic effects the filtrate of the coccus is superior to the broth in which it is grown. According to Besredka the antivirus is a heat-stable body elaborated by growth of the staphylococcus and tending to inhibit further growth of the same coccus but not of other organisms. Schweinburg employed *B. coli* filtrates in furunculosis with good results. He showed, also, that when *B. typhosus*, *B. coli*, *Staphylococcus aureus*, or *Streptococcus pyogenes* were cultivated in the filtrates of these organisms, there was no evidence of specificity even though the pH of all filtrates was carefully adjusted to the original value of 7.5. There comes a time, he says, when nothing will grow in these filtrates. He cannot exclude the presence of some body in filtrates which acts non-specifically on all organisms, and he comments in this connection on the occasional rapid death when filtrates are freshly seeded—a death which is prevented by a little added broth.

With regard to anthrax infection, Besredka deduces from his experimental work that the skin is the only susceptible portion of the guinea-pig's anatomy, and, further, that if the skin is immunized, as he claims it can be, by inunction of the Pasteurian attenuated vaccines, the whole body can be rendered solidly immune to an otherwise lethal dose of living virulent

culture. The fact that the site of inoculation of an organism is of prime importance for successful infection has long been known, and so far as anthrax is concerned it was noted many years ago by Noetzel (1898) and van Leent (1900) that the intraperitoneal inoculation of anthrax necessitated for successful infection a much greater dose than the subcutaneous, and the same has been postulated of the intravenous route. Besredka would go further and claim that if a general infection occurred after intraperitoneal, intramuscular or intravenous inoculation, the reason was to be sought in an inadvertent injury of the skin produced by the operative procedure. The thesis has not withstood criticism. Careful experiments of a quantitative nature have been made in which any possible pollution of the external skin has been avoided. Possibly the most worthy of record of such experiments are those of Sobernheim and Murata (1924), who showed clearly that the dose necessary for infection was highest by the intraperitoneal and intravenous routes and lowest for the intramuscular route. Between these extremes came the intracutaneous and subcutaneous doses, which differed little from each other. The doses were of this order: intravenous, 1 in 10,000; intraperitoneal, 1 in 50,000; subcutaneous, 1 in 1,000,000; intracutaneous, 1 in 1,000,000; intramuscular, < 1 in 1,000,000. There would appear to be no doubt, therefore, that the dose necessary for successful infection varies with the local defence, and so far as the skin is concerned the experiments lend no support to Besredka's thesis that it constitutes the only vulnerable tissue. In man, at least, it is well known that the skin may effectively localize an anthrax infection and prevent blood invasion. It may be recalled also that the inunction of the guinea-pig's skin with *B. pestis* is on the whole not so certain in producing infection as the direct introduction into the subcutis or muscle, however valuable the inunction method may be in other spheres of plague investigation. The varying susceptibilities of different regions of the body to different infections is discussed in papers by Uchida (1926) and Lange and Gutdeutsch (1928), and it would appear to be established that a favourable spot for infection in one species of animal may prove to be an unfavourable one in another, even for the same infection.

Bautz (1929), in an interesting series of experiments on anthrax infection in the guinea-pig and methods of producing solid immunity in that animal—a notoriously difficult matter—agrees with Besredka that the inunction of the skin with the attenuated Pasteurian vaccines is superior to any method involving the subcutaneous inoculation of antigen, but he would explain the difference simply on the ground that the skin is more tolerant and that consequently a greater opportunity is afforded for successful immunization leading to solid immunity without incidental casualties. Bautz shows clearly that as immunization of guinea-pigs by inunction of the attenuated vaccines (I and II) proceeds, there is observed not only a very gradual development of immunity as tested by subcutaneous inoculation of the second attenuated vaccine, but a still more gradual

development of immunity as tested by subcutaneous inoculation of virulent anthrax. The latter form of solid immunity is, indeed, only rarely realizable. The whole process of immunization on Besredka's lines in no way suggests that the local immunization of the one and only vulnerable site is the one thing needful for solid immunity, but rather that the persistent application of antigen to a relatively tolerant site may ultimately lead to a general immunity of the tissues. In natural immunity we are familiar with many instances of local susceptibilities and local immunities combined in any one host whether that host is generally regarded as naturally immune or naturally susceptible. The rat, for example, is resistant to diphtheria bacilli whether introduced into the skin or peritoneum. To the organism of Preisz-Nocard, however—an organism closely allied to *C. diphtheriae*—the rat is completely resistant only when the organism is introduced into the skin or subcutis. When it is introduced into the peritoneum, the animal regularly succumbs from a blood invasion (Ledingham, 1926).

With regard, finally, to Besredka's experiments on immunization of the intestinal epithelium by oral administration of killed paratyphoid or dysentery bacilli preceded by exhibition of bile, it cannot be said at present that when solid immunity results, as it may do, the effect is to be attributed to a 'desensibilization' of the intestinal epithelium, the one susceptible tissue. Nor is antibody-development following oral administration of killed antigen in abeyance, as Besredka alleges (Kanai, 1921-2; Otten and Kirschner, 1927, &c.), so that the resultant immunity is not one in which antibodies can be said to play no part. The relative efficacy of oral and subcutaneous methods of introducing antigen are discussed elsewhere (see Chapter III).

In concluding this section on local immunity it is fitting to acknowledge the stimulus that Besredka's thesis has given to research on local defence mechanisms in skin or elsewhere, though in hardly a single particular can it be said that the novel views he put forward as to the mechanism and extent of the local immunity observed by him have been accepted. It would appear, however, to be definitely established that the skin can be so treated by specific and non-specific means as to withstand and localize specific infections of low virulence. Where specific infections of high virulence for the host are concerned, only the specific antigen would seem to be effective as a localizing agent, as in Grumbach's experiments cited above. Even here, however, there is no evidence of solid immunity as tested by introduction of the virulent test organism elsewhere than in the prepared skin area. A discussion of the cellular processes underlying both local and general defence mechanisms will follow in the section dealing with the reticulo-endothelial system. Here I may simply refer to the description by Grumbach (1929) of the histology of the dermis after inoculation of filtrate containing 'antivirus'. There is in the *stratum subreticulare* a very definite zone of reactive tissue. Not only are the cell elements involved but also the lymph and blood channels. This zone

overlying the skin muscularis is that in which reactive changes are most prominent when living viruses or 'blockading' substances are introduced into the dermis.

### The Reticulo-Endothelial System in relation to Problems of Natural Immunity.

We have frequently found it necessary to allude to the activities of the reticulo-endothelial system in attempting to form an ultimate analysis of problems of local and general defence. At the outset it cannot be too strongly emphasized that the conception of a system of cells characterized by their capacity to take up from the blood-stream foreign material, whether organized bacterial protein or dyestuffs, is but an extension of Metchnikoff's fundamental work on comparative phagocytic powers of the more mobile cells of the body, the polynuclears and the mononuclears with their respective micro- and macrocytases. The nucleus of the reticulo-endothelial system as at present defined is to be found in the importance attached by Metchnikoff to the activities of mononuclear cells in various infective and non-infective processes natural or induced. Aschoff arranges the cells of the reticulo-endothelial system in the following order, commencing with those whose capacity to take up dyes is least marked : (1) endothelia of blood- and lymph-vessels ; (2) connective tissue-cells or fibrocytes ; (3) cells of the splenic and lymphatic reticulum ; (4) reticulo-endothelial cells of the lymph sinuses, the blood sinuses of spleen, of liver capillaries (Kupffer cells), of bone-marrow, adrenal cortex and pituitary ; (5) histiocytes or mobile cells of connective tissue ; and (6) splenocytes or monocytes of the circulating blood. This system of cells is accordingly confined to no single organ, but there is no doubt that one organ, viz. the spleen, contains a predominating proportion. That organ has borne the brunt of most of those experimental procedures which have been devised to throw this system out of action in the attempt to illuminate problems concerned with the cellular sources of antibody production in the normal and immunized animal, the ultimate analysis of general and local defence mechanisms and a host of other problems. It may be said here that these attempts to influence the reticulo-endothelial system by blockade or splenectomy or both combined have laid bare potentialities for defence on the part of these cells which have been in the past ignored or obscured by an over-concentration during the past two decades on the immune properties residing in the body fluids. Metchnikoff, it may be recalled, was concerned to defend against the inroads of the humoralists his theory of phagocytosis as the all-important defence mechanism and to attribute the very definite defences residing in the body fluids to an origin in cell ferments. In his view the phagocytic cells of the immunized animal received a special impression, the result of which could be demonstrated *in vitro*. With the appearance on the scene of opsonins and tropins a *modus vivendi* seemed to be established between the purely cellular and the purely humoral theories in relation

to phagocytosis, but it has not been till quite recent years that a more intimate relationship has been recognized between cells and fluids as the result of histological and experimental studies on the response of the reticulo-endothelial cells to foreign protein and dyes introduced either into the blood-stream or into local sites such as the subcutaneous tissue and the dermis. The capacity of these cells to respond to foreign protein, not only by phagocytosis leading perhaps to antibody formation but also by active proliferation, would appear to be essential attributes. Further, it would seem that long after the stimulus is over, these cells retain the capacity for accelerated proliferation in response to fresh stimuli of the same or even of different kind. In this section I propose to summarize the evidence with regard to the influence of blockade and splenectomy, or both, on certain processes of natural immunity.

#### *The Disposal of Organisms Introduced into the Blood-Stream.*

Since Wyssokowitsch's (1886) early work on this subject it has been believed that the disappearance from the blood of organisms injected into it or gaining entrance to it from another site is due to their being taken up by phagocytes in the lungs and other viscera and there disposed of. The phenomena which accompany this process have been studied recently by various workers, of whom I may mention Ørskov, Jensen and Kobayashi (1928) and H. D. Wright (1927). In the case of pneumococci it would appear that after entrance into the blood-stream of rabbits they disappear rapidly only to appear again in it, causing a secondary septicæmia, from which death may result. In the specifically immunized animal, on the other hand, injected pneumococci disappear quickly from the blood-stream and there is no secondary invasion. The organisms are almost certainly disposed of by phagocytes in lungs and other organs, and in this phagocytosis the cells of the reticulo-endothelial system have been shown to play a predominant role. The effect of splenectomy on this disposal of foreign protein has been studied in various affections, bacterial and protozoal. In the case of *Bartonella muris*, its very demonstration appears to depend on the defence rupture induced by splenectomy. Hoen, Tschertkow and Zipp (1926) noted a fairly regular appearance of vaccine virus in the blood after splenectomy and blockade, while Goldmann (1927-8) obtained evidence in the blockaded guinea-pig of the presence of this virus in the blood on the second day after infection. Vaccine virus had not previously been demonstrated in the blood of guinea-pigs after cutaneous infection. It was also of interest in Goldmann's experiments that in the blockaded animals the skin reaction to vaccinia was definitely accelerated and of reduced duration. This he would attribute to the escape of virus into the circulation, thus reducing the quantum available in the blocked site for local proliferation. It would seem more probable, however, that the acceleration was correlated with the increased reactivity of the local reticulo-endothelial cells. As we shall see later, the two-phase character of the response of reticulo-endothelial tissue to blockade may

profoundly affect the nature of the response. The influence of splenectomy and blockade on the course of experimental recurrens infection has been fairly extensively studied (Kritschewski and collaborators, 1926, 1927, 1928; Jungeblut and collaborators, 1926<sup>1 & 2</sup>, 1927<sup>1 & 2</sup>; Feldt and Schott, 1927). There would appear to be considerable evidence that recurrens infection in splenectomized mice runs a very disastrous course, though more recently Feldt and Eisenmenger (1928), taking special precautions to avoid the use of mice liable to intercurrent infections and also to avoid undue prolongation of the splenectomy operation, have claimed that the course of the infection is not necessarily more severe than in normal mice. Regendanz and Kikuth (1927) find that the infection of rats with *T. lewisi* after splenectomy runs a severe course and may end fatally. On the other hand, in connection with chemotherapeutic experiments in splenectomized animals (see later), Kritschewski and his collaborators do not find that the spleen, or the reticulo-endothelial system generally, plays the part of a protective mechanism in experimental trypanosome infections as it appears to do in recurrens infection. Splenectomized animals inoculated with trypanosomes react in a manner not dissimilar to that of normal animals with intact reticulo-endothelial system.

When we come to consider bacterial infections in normal animals we find that blockade or splenectomy or both exert little influence on the course of the disease. The course of Breslau (Aertrycke) infection in mice, for example (Ørskov, Jensen and Kobayashi, 1928), or of streptococcus infection in mice (Meersohn, 1927-8), is little affected by splenectomy and blockade. Nor has it been possible by these procedures to affect materially the natural immunity of certain species to anthrax infection. It may be recalled that so long ago as 1889 Bardach claimed to have reduced the natural immunity of the dog to anthrax by removing its spleen. Recent work designed to produce similar ruptures of the naturally immune state by blockade methods have not been very successful. Collon (1927) failed to break the hen's immunity to anthrax in this way, though Singer (1925) claimed to have done so. It is probable that the insult of blockade and splenectomy is insufficient to break a natural immunity owing to the rapidity with which compensatory mechanisms in the naturally immune animal become established. Moreover, in the naturally immune state there may be a much wider distribution of the protective tissue than in the actively immunized animal, and consequently one would expect that in the latter animal, whose reticulo-endothelial cells have been specially trained for a special purpose, the chances of a rupture of this fine mechanism by blockade and splenectomy would be much greater. This, indeed, appears to be the case. Singer and Adler (1924) find that the usual prompt disappearance of pneumococci from the blood which takes place in the immunized animal fails to occur in the blockaded animal (see also Meyer, 1926; Neufeld and Meyer, 1924). The rupture is, however, only a temporary one. Similarly in mice, splenectomy and blockade reduced the immunity resulting from swine erysipelas immunization as tested by

a dose of virulent culture (Bieling, 1929). In connection with antitoxic immunity, Jungeblut and Berlot (1926) blockaded guinea-pigs with Indian ink and thereafter immunized them with toxin-antitoxin mixtures. The result was only a delay in the production of antitoxin. By the fourth week the titres of the blocked and unblocked animals approximated. On the elimination of a foreign serum blockade does not appear to have any effect (Collon, 1927), nor is precipitin-production in response to it affected.

#### *Antibody Formation.*

Among the earliest workers on this subject were Bieling and Isaac (1922). The latter found that blockade combined with splenectomy hindered the formation of haemolysin in response to injection of foreign blood. The action on complement was investigated by Jungeblut and Berlot (1926), who inoculated guinea-pigs intravenously with ink and thereafter tested the complement titre at intervals from 15 minutes to 4 days. A drop in titre occurred, reaching its lowest level 3 hours after injection, but in 24 hours there was a complete return to normal. Possibly the effect on complement had to do with its mechanical adsorption by the Indian ink. Gay and Clark (1924) also found that blockade of rabbits with trypan blue hindered completely the formation of haemolysin and vibriolysin, while Siegmund (1922) noted inhibition of agglutinin and lysin production. On the other hand, Standenath (1923) observed an increase in the formation of precipitins after blockade. Discrepancies such as these are almost certainly due, as Motohashi (1922) suggests, to the fact that reticulo-endothelial tissue is temporarily paralysed by a small dose of antigen but stimulated to active proliferation by a large dose (see below). More recently Kobayashi (1926) has recorded data on agglutinin development in splenectomized mice inoculated intravenously with killed typhoid bacilli. In the various tests the spleen was removed two days before injection, one month before injection and two days after injection. In all these cases practically no agglutinin formation was noted in comparison with control mice, which yielded a titre reaching 1 in 200. No compensation for loss of spleen was afforded by subcutaneous or intra-peritoneal implantation of spleen fragments. As the injections of typhoid bacilli were repeated, agglutinin formation came up to the level of the controls. Even combined splenectomy and blockade resulted only in a temporary arrest of agglutinin production.

The study by Stewart and Parker (1926) on the effect of collargol blockade in rabbits and guinea-pigs yielded curiously irregular results. In guinea-pigs, precipitin formation was reduced, but the agglutinins might be increased or diminished. In rabbits, depression of precipitin formation as also of agglutinins and lysins was noted. It is quite clear from the data cited that the effects of blockade and splenectomy or both on antibody development are apt to be irregular, but on the whole there would appear to be little doubt that the first effect, at least, of such

interferences is an arrest of antibody development, due in all probability to depression of the capacity of reticulo-endothelial cells to elaborate antibody in the midst of their preoccupation with the phagocytosis of dyestuffs. It is now, however, well recognized that the injection of a dye (e.g. in the dermis) is followed not only by active phagocytosis of the dye by the available macrophages which are rapidly mobilized, but by a subsequent massive proliferation of reticulo-endothelial tissue in the neighbourhood of the dye. This phenomenon was clearly evident in the writer's experiments on the effect of local blockade on the reaction to vaccinia (Ledingham, 1927). Such stimulation to activity would naturally lead to increased elaborative powers by reticulo-endothelial tissue in dealing with antigen and consequently to increased antibody production. There is a consensus of opinion that the balance between depression and stimulation of reticulo-endothelial tissue induced by the blockading dyestuff determines the resultant yield of antibody in response to antigen or alternatively the resultant local defence.

Leites and Riabow (1927) conclude from their experiments on the elimination of colloidal iron that the reaction to blockade as tested by the content of iron in the circulating blood takes two phases, (1) in which the phagocytosis of the dye by reticulo-endothelial cells is diminished, leading to a greater concentration of the dye in the blood, and (2) in which there has been an activation of the reticulo-endothelial tissue with a corresponding diminution in the iron-content of the blood. The intensity of the blockade or dose of blockading substance had an important bearing, therefore, on the final result.

#### *Site of Antibody Formation.*

This question is now intimately bound up with that of the functional activity of the reticulo-endothelial system. It has long been believed, though exact proof has not been forthcoming, that the main site of antibody formation is the spleen and lymphoid organs, and it has been usual to date this conception from the work of Pfeiffer and Marx (1898), who found that antibodies were discernible in extracts of lymphoid organs before they were demonstrable in the blood-stream. The employment in recent years of tissue-culture methods, dating from Carrel and Ingebrigtsen's (1912) work, has, I think, shown clearly that antibody development can take place in cultures of mammalian reticulo-endothelial tissue which before seeding have been removed from animals inoculated intravenously with a dose of antigen. Meyer and Loewenthal (1927-8), who review this subject, supply data of their own which would appear to prove fairly conclusively that in cultures of mesenteric fragments from animals inoculated intravenously with killed typhoid bacilli, and containing only reticulo-endothelial tissue, agglutinins develop. The titre was admittedly low and it reached its height in three days. After the fifth day agglutinins were not demonstrable, a fact which the authors would attribute to a dedifferentiation of tissue in the course of growth.

In the attempt to solve this question use has also been made of perfusion experiments. Olsen (1922), for example, showed that a serum-free guinea-pig liver can dissolve sensitized red cells passed through it, especially if small amounts of end-piece and mid-piece which do not lyse *per se* are added. When the third component of complement is passed through a serum-free organ the sensitized cells get all they require for lysis. The results suggested that complement-components are secreted from organ cells during the perfusion, as neither mid-piece nor end-piece exist in the pressed juice of the serum-free liver. Other methods of attack, such as the rendering of animals aleucocytic by thorium X (Lippmann and Plesch, 1913) and various other means which cannot be cited here, have not yielded data which suggest an origin of complement from leucocytes. More information on this subject is to be expected from further researches on the reticulo-endothelial system and tissue-culture experiment. Recent experiments by Topley (1930) would suggest that, after inoculation of bacterial antigen into animals, it is fixed by the spleen, which, however, rapidly reduces it to a form not recognizable as ordinary antigen if one may judge from the effect on agglutinin-titre of introducing the spleen tissue into normal animals.

#### *Blockade of the Reticulo-Endothelial System and Virulence.*

By blockading guinea-pigs and rabbits Fialho and Pacheco (1928) claim to have rendered an otherwise avirulent strain of *B. pestis* virulent for these animals, but the data are not carefully documented.

#### *Blockade and Chemotherapy.*

It has been shown by a number of workers in recent years that splenectomy and blockade have a profound effect on the action of drugs in trypanosome and recurrens infections, and the view has been put forward that the drug acquires its truly parasitotropic action only when it has been elaborated by the cells of the reticulo-endothelial system. It is impossible here to detail the data on this subject by Kritschewski, Meersohn, Feldt and Schott, Jungeblut, Rubinstein, Lisgunova *et al.*, and those specially interested may be referred to abstracts of this literature in the *Tropical Diseases Bulletin*.

In any case the claim that the action of a chemotherapeutic drug in these infections depends on the intactness of the reticulo-endothelial system has by no means been generally accepted. It would seem more probable from recent work of Kikuth and Regendanz (1929) that the apparent lack of potency of the drug in splenectomized mice is due not to removal of the spleen as such but to the interference with normal defence mechanisms caused by the operative procedure. In larger animals such as guinea-pigs and rabbits, in which the spleen from its relatively small size would appear to be of less importance to the general economy than in rats and mice, its removal does not affect the course of a subsequent protozoal infection nor is the activity of a drug impaired.

**Phenomena illustrating the use of 'Tissue Depressants' in  
Promoting Virus or Bacterial Action.**

It would seem appropriate here to refer to certain phenomena attributable to the action of what are known as tissue depressants. These substances have been studied principally in connection with the germination of spores of anaerobes when introduced into the subcutaneous tissues. It has been shown that substances like quinine, lactic acid, calcium salts, &c., facilitate by virtue of their necrotizing action on local tissue the germination in the body of spores of *B. tetani* and gas-gangrene organisms (Semple, 1911; Bullock and Cramer, 1919). All that we can definitely say at present is that the milieu produced by the tissue depressant supplies the conditions essential for germination of spores. If the depressant is introduced at one spot and the tetanus spores at another, it has been shown that tetanus may ensue owing to the germination of spores which have been transported to the depressed area. The recent important work of Fildes (1929) would suggest that so far as the germination of tetanus spores in the body is concerned, at least one essential factor is the presence in the milieu of an appropriate oxidation-reduction potential. If tetanus spores are unable to germinate in the guinea-pig subcutis owing to the unsuitability of the environment in this respect, as Fildes suggests, the necrotic action of calcium on tissue would bring about the alteration of eH necessary for germination.

In virus studies it has long been known that local irritation of skin sites favours not only the proliferation of virus implanted thereon but also the settlement in such areas of virus introduced into the blood-stream. Various theories have been put forward to explain this phenomenon. Levaditi, in the case of vaccinia, suggested that the induced activity as evidenced by mitoses, of the basal epithelia, favoured the settlement of virus in these cells, for which, according to him, it had a special affinity. This view has not received much support. The writer concluded from his experimental data that the reticulo-endothelial system of the part was mainly involved in the interaction between host and virus, and that the irritation of the skin paved the way for the deposition and proliferation of virus in the dermis, by opening up access to dermal tissue. It is clear, however, that other factors must play a part if we are to explain the predilection of virus for skin (variola, varicella, &c.). The writer suggested that the low oxygen tension in the dermis possibly favoured deposition of virus, and it has been shown (Ledingham and McClean, 1928) that a virus adapted to growth in dermis may lose much of its capacity for proliferation when placed on the scarified skin. It is possible that further work along the lines studied by Fildes may throw light on this problem.

Findlay (1928) has published data which would suggest that bodies of the histamine class may be those primarily concerned in facilitating the deposition of viruses and bacteria in irritated skin spots after introduction into the blood-stream. Whether such bodies are elaborated in the natural

disease by action of virus on host is not known, but it was definitely shown that the intradermal injection of histamine far more greatly favoured the proliferation at the site of injection of intravenously introduced viruses and bacteria than simple phosphate buffer solutions. The function of the histamine would be to produce local capillary dilatation and to increase the permeability of endothelia, thus favouring local deposition of virus. Recently, also, it has been shown by Duran Reynals (1928) that the inoculation of tissue extracts (testicular, renal, &c.) along with vaccine virus, staphylococci, &c., leads to a noteworthy enhancement of the local dermal response to these agents.

## REFERENCES.

ARKWRIGHT, J. A. & ZILVA, S. S., 1924, *J. Path. Bact.*, **27**, 346.  
 BAILEY, G. H., 1928, *The Newer Knowledge of Bacteriology and Immunology*, ed. Jordan and Falk, Chicago Univ. Press ; 1929, *Amer. J. Hyg.*, **9**, 192.  
 BARDACH, J., 1889, *Ann. Inst. Pasteur*, **3**, 577.  
 BARG, G. S., 1928, *Zbl. Bakt.*, Abt. I, Orig., **108**, 341.  
 BARREAU, E., 1909, *Arch. Hyg.*, Berl., **70**, 331.  
 BAUTZ, TH., 1929, *Z. ImmunForsch.*, **61**, 115.  
 BEDSON, S. P., 1915, *J. Path. Bact.*, **19**, 191.  
 BESREDKA, A., 1919, *Ann. Inst. Pasteur*, **33**, 301, 557, 882 ; 1921, *ibid.*, **35**, 421 ; 1924, *ibid.*, **38**, 565.  
 BESSEMANS, A. & NÉLIS, P., 1928, *C.R. Soc. Biol.*, Paris, **99**, 329.  
 BESSEMANS, A. & SELDESLAGHTS, A., 1928<sup>1</sup>, *C.R. Soc. Biol.*, Paris, **99**, 628 ; 1928<sup>2</sup>, *Ann. Soc. sci. Brux.*, Ser. C, **48**, 121.  
 BIELING, R., 1923, *Z. ImmunForsch.*, Tl. I, Orig., **38**, 193 ; 1925<sup>1</sup>, *Z. Hyg. InfektKr.*, **104**, 518 ; 1925<sup>2</sup>, *ibid.*, **104**, 631 ; 1926, *ibid.*, **105**, 254 ; 1929, *Zbl. Bakt.*, Abt. I, Orig., Beiheft **110**, 195.\*  
 BIELING, R. & ISAAC, S., 1922, *Z. ges. exp. Med.*, **28**, 180.  
 BOYCOTT, A. E. & PRICE-JONES, C., 1926, *J. Path. Bact.*, **29**, 87.  
 BRAUN, H. & NODAKE, R., 1924, *Zbl. Bakt.*, Abt. I, Orig., **92**, 429.  
 BROWNING, C. H., GULBRANSEN, R. & RUSS, S., 1919, *J. Path. Bact.*, **23**, 127.  
 BUCHNER, 1889, *Zbl. Bakt.*, **5**, 817 ; **6**, 1.  
 BULL, C. G., 1915, *J. Exp. Med.*, **22**, 457, 466, 475, 484.  
 BULLOCK, W. E. & CRAMER, W., 1919, *Proc. Roy. Soc.*, B, **90**, 513.  
 CALMETTE, A., 1895, *Ann. Inst. Pasteur*, **9**, 225.  
 CARREL, A. & INGEBRIGTSEN, R., 1912, *J. Exp. Med.*, **15**, 287.  
 COBBETT, L., 1899, *Brit. Med. J.*, i, 902.  
 COBBETT, L. & MELSMORE, W. S., 1896, *J. Path. Bact.*, **3**, 39.  
 COCA, A. F., RUSSELL, E. F. & BAUGHMAN, W. H., 1921, *J. Immunol.*, **6**, 387.  
 COLE, R. I., 1904, *Z. Hyg. InfektKr.*, **46**, 371.  
 COLEBROOK, L., EIDENOW, A. & HILL, L., 1924, *Brit. J. Exp. Path.*, **5**, 54.  
 COLLON, N. G., 1927, *C.R. Soc. Biol.*, Paris, **98**, 427.  
 COMBIESCO, D., 1928<sup>1</sup>, *Arch. Roumaines Path. Exp. Micro.*, **1**, 81 ; 1928<sup>2</sup>, *ibid.*, **1**, 341.  
 DAVESNE, J., 1927, *C.R. Soc. Biol.*, Paris, **97**, 1061.  
 DEAN, G., 1905, *Proc. Roy. Soc.*, B, **76**, 506 ; 1907<sup>1</sup>, *ibid.*, B, **79**, 350 ; 1907<sup>2</sup>, *Brit. Med. J.*, ii, 1409.  
 DENYS, J., 1898, *Zbl. Bakt.*, Abt. I, **24**, 685.  
 DENYS, J. & LECLEF, J., 1895, *Cellule*, ii, 177.  
 DREYER, G. & WALKER, E. W. A., 1909, *J. Path. Bact.*, **14**, 28.  
 DUNLOP, E. M., 1928, *J. Path. Bact.*, **31**, 769.  
 DURAN REYNALS, F., 1928, *C.R. Soc. Biol.*, Paris, **99**, 6.  
 FELDT, A. & EISENMAYER, C., 1928, *Z. Hyg. InfektKr.*, **109**, 410.  
 FELDT, A. & SCHOTT, A., 1927, *Z. Hyg. InfektKr.*, **107**, 453.  
 FELIX, A., 1929, *J. Hyg., Camb.*, **28**, 418.  
 FELIX, A. & OLITZKI, L., 1929, *Brit. J. Exp. Path.*, **10**, 26.

FENN, W. O., 1921<sup>1</sup>, *J. Gen. Physiol.*, **3**, 575; 1921<sup>2</sup>, *ibid.*, **3**, 439; 1922<sup>1</sup>, *ibid.*, **4**, 331; 1922<sup>2</sup>, *ibid.*, **5**, 169; 1928, *The Newer Knowledge of Bacteriology and Immunology*, ed. Jordan and Falk, Chicago Univ. Press, p. 861.

FIALHO, A. & PACHECO, G., 1928, *C.R. Soc. Biol.*, Paris, **98**, 1562.

FILDES, P., 1929, *Brit. J. Exp. Path.*, **10**, 151, 197.

FINLAY, G. M., 1923, *J. Path. Bact.*, **28**, 1, 485; 1928, *ibid.*, **31**, 633.

FLEMING, A., 1922, *Proc. Roy. Soc., B*, **93**, 306; 1924, *ibid.*, **96**, 171; 1926, *Brit. J. Exp. Path.*, **7**, 274; 1928, *Proc. Roy. Soc. Med.*, **21**, 25, Sect. Otol.; 1929, *Lancet*, Lond., i, 217.

FLEMING, A. & ALLISON, V. D., 1922, *Proc. Roy. Soc., B*, **94**, 142; 1927, *Brit. J. Exp. Path.*, **8**, 214.

FRIEDBERGER, E., BOCK, G. & FÜRSTENHEIM, A., 1929, *Z. ImmunForsch.*, **64**, 294.

GAY, F. P., 1923, *J. Immunol.*, **8**, 1.

GAY, F. P. & CLARK, A. R., 1924, *J. Amer. Med. Ass.*, **83**, 1296.

GAY, F. P. & MORRISON, L. F., 1921, *J. Infect. Dis.*, **28**, 1; 1923, *ibid.*, **33**, 338.

GAY, F. P. & STONE, R. L., 1920, *J. Infect. Dis.*, **26**, 265.

GIBSON, H. J., 1930, *J. Hyg., Camb.*, **30**, 337.

GLYNN, E. E. & COX, G. LISSANT, 1909, *J. Path. Bact.*, **14**, 90.

GOLDMAN, A., 1928, *Zbl. Bakt.*, Abt. I, Orig., **105**, 333.

GORDON, J. & WORMALL, A., 1928, *J. Path. Bact.*, **31**, 753.

GREEN, H. N. & MELLANBY, E., 1928, *Brit. Med. J.*, ii, 691; 1930, *Brit. J. Exp. Path.*, **11**, 81.

GRUBER, M. & FUTAKI, K., 1907, *Münch. med. Wschr.*, **54**, 249.

GRUMBACH, A., 1928, *Z. ImmunForsch.*, **57**, 357; 1929, *Zbl. Bakt.*, Abt. I, Orig., **110**, Beiheft 146.\*

HAHN, M., 1895, *Arch. Hyg.*, Berl., **25**, 105.

HEINBECKER, P. & IRVINE-JONES, E. I. M., 1928, *J. Immunol.*, **15**, 395.

HEKTOEN, L., 1911, *J. Amer. Med. Ass.*, **57**, 1579.

HIRSZFIELD, L., 1924, *Klin. Wschr.*, **3**, 1180, 1308, 2085.

HIRSZFIELD, L. & SEYDEL, J., 1925, *Z. Hyg. InfektKr.*, **104**, 465, 478.

HOEN, E., TSCHERTKOW, L. & ZIPP, W., 1926, *Z. Hyg. InfektKr.*, **106**, 624.

HYDE, R. R., 1923, *J. Immunol.*, **8**, 267.

ISSAEFF, 1894, *Z. Hyg. InfektKr.*, **18**, 287.

JAGGI, M., 1923, *Z. ImmunForsch.*, Tl. I, Orig., **36**, 482.

JUNGEBLUT, C. W., 1927<sup>1</sup>, *J. Exp. Med.*, **48**, 609; 1927<sup>2</sup>, *Z. Hyg. InfektKr.*, **107**, 357.

JUNGEBLUT, C. W. & BERLOT, J. A., 1926<sup>1</sup>, *J. Exp. Med.*, **43**, 613; 1926<sup>2</sup>, *ibid.*, **43**, 797.

KANAI, S., 1921, *Brit. J. Exp. Path.*, **2**, 256; 1922, *ibid.*, **3**, 158.

KIKUTH, W. & REGENDANZ, P., 1929, *Z. ImmunForsch.*, **61**, 422.

KLEIN, E., 1893, *Brit. Med. J.*, i, 632.

KOBAYASHI, K., 1926, *C.R. Soc. Biol.*, Paris, **94**, 599.

KRAUS, R., 1927, *Handb. d. path. Mikroorg.*, hrsg. Kolle, Kraus u. Uhlenhuth, Jena, **3**, 1.

KRITSCHEWSKI, I. L., 1927, *Zbl. Bakt.*, Abt. I, Orig., **104**, Beiheft 214\*; 1928, *Z. ImmunForsch.*, **59**, 1.

KRITSCHEWSKI, I. L. & MEERSOHN, I. S., 1926, *Z. ImmunForsch.*, **47**, 407.

KRITSCHEWSKI, I. L. & RUBINSTEIN, P. L., 1927, *Z. ImmunForsch.*, **51**, 27.

KRITSCHEWSKI, I. L. & SCHAPIRO, S., 1928, *Z. ImmunForsch.*, **58**, 308.

KRITSCHEWSKI, I. L. & SCHWARZMANN, 1928, *Z. ImmunForsch.*, **58**, 322.

KYES, P., 1916, *J. Infect. Dis.*, **18**, 277.

LANGE, B. & GUTDEUTSCH, H., 1928, *Z. Hyg. InfektKr.*, **109**, 253.

LASCHTSCHENKO, P., 1909, *Z. Hyg. InfektKr.*, **64**, 419.

LEDINGHAM, J. C. G. & BULLOCH, W., 1906, *Studies in Pathology*, Aberd. Univ. Press.

LEDINGHAM, J. C. G., 1907, *Proc. Roy. Soc., B*, **79**, 482; 1908, *ibid.*, **80**, 188; 1909, *Z. ImmunForsch.*, Tl. I, Orig., **3**, 119; 1912, *J. Hyg., Camb.*, **12**, 320; 1922, *Lancet*, Lond., ii, 898; 1923-4, *Trans. Soc. Trop. Med. Hyg.*, Lond., **17**, 357; 1926, *J. State Med.*, **34**, 125; 1927, *Brit. J. Exp. Path.*, **8**, 12.

LEDINGHAM, J. C. G. & DEAN, H. R., 1912, *J. Hyg., Camb.*, **12**, 152.

LEDINGHAM, J. C. G. & McCLEAN, D., 1928, *Brit. J. Exp. Path.*, **9**, 216.

VAN LEENT, J. B., 1900, *Zbl. Bakt.*, Abt. I, **28**, 737.

LEITES, S. & RIABOW, A., 1927, *Z. ges. exp. Med.*, **58**, 314.

LEVINTHAL, W., 1929, *Z. Hyg. InfektKr.*, **110**, 182.  
 LIPPmann & PLESCH, 1913, *Z. ImmunForsch.*, Tl. I, Orig., **17**, 548.  
 LISGUNOVA, A. W., 1928<sup>1</sup>, *Z. ImmunForsch.*, **57**, 292; 1928<sup>2</sup>, *ibid.*, **58**, 93.  
 LISGUNOVA, A. W. & BUTJAGINA, A. P., 1927, *Z. ImmunForsch.*, **51**, 56.  
 LISTER, JOSEPH, 1880, *The Collected Papers of Joseph, Baron Lister*, Vol. I, Clarendon Press, Oxford.  
 LÖWENBERG, W., 1928, *Z. Hyg. InfektKr.*, **108**, 1.  
 MACKIE, T. J. & FINKELSTEIN, M. H., 1930, *J. Hyg., Camb.*, **30**, 1.  
 MADSEN, T. & WATABIKI, T., 1919, *Medd. k. VetenskAkad. Nobelinst.*, **5**.  
 MADSEN, T. & WULFF, O., 1916, *Overs. danske Vidensk. Selsk. Forh.*, p. 339.  
 MALLORY, T. B. & MARBLE, A., 1925, *J. Exp. Med.*, **42**, 465.  
 MALONE, R. H., AVARI, C. R. & NAIDU, B. P. B., 1925, *Ind. J. Med. Res.*, **13**, 121.  
 MARCHAND, L., 1898, cited by DENYS, *Zbl. Bakt.*, Abt. I, **24**, 685.  
 MEERSOHN, J., 1927-8, *Z. ImmunForsch.*, **54**, 313.  
 MENNES, F., 1897, *Z. Hyg. InfektKr.*, **25**, 413.  
 MÉTALNIKOV, S. & CHORINE, V., 1926, *Ann. Inst. Pasteur*, **40**, 893.  
 MEYER, H., 1926, *Z. Hyg. InfektKr.*, **108**, 124.  
 MEYER, H. & LOEWENTHAL, H., 1927-8, *Z. ImmunForsch.*, **54**, 409.  
 MILLER, C. P., JR., 1927, *Z. Hyg. InfektKr.*, **107**, 253.  
 MOORE, H. D., 1919, *J. Immunol.*, **4**, 425.  
 MOTOHASHI, S., 1922, *J. Med. Res.*, **43**, 419.  
 MOTTRAM, J. C. & KINGSBURY, A. N., 1924, *Brit. J. Exp. Path.*, **5**, 220.  
 MOXTER, DR., 1899, *Zbl. Bakt.*, Abt. I, **28**, 344.  
 MUDD, S., LUCKÉ, B., McCUTCHEON, M. & STRUMIA M., 1929, *J. Exp. Med.*, **49**, 779, 797.  
 MUIR, R. & MARTIN, W. B. M., 1906, *Brit. Med. J.*, ii, 1783; 1907, *Proc. Roy. Soc., B*, **79**, 187.  
 NAKAHARA, W., 1925, *J. Exp. Med.*, **42**, 201.  
 NEUFELD, F. & HÜNE, 1905, *Zbl. Bakt.*, Abt. I, Orig., **38**, 456; 1907, *Arb. GesundhAmt.*, Berl., **25**, 164.  
 NEUFELD, F. & MEYER, H., 1924, *Z. Hyg. InfektKr.*, **103**, 595.  
 NEUFELD, F. & RIMPAU, W., 1904, *Deuts. med. Wschr.*, **30**, 1458.  
 NOETZEL, W., 1898, *Arch. klin. Chir.*, **57**, 311.  
 NUTTALL, G. H. F., 1888, *Z. Hyg.*, **4**, 353.  
 OLSEN, O., 1922, *Biochem. Z.*, **133**, 24.  
 ØRSKOV, J., JENSEN, K. A. & KOBAYASHI, K., 1928, *Z. ImmunForsch.*, **55**, 34.  
 OSBORNE, T. B., MENDEL, L. B. & FERRY, EDNA, L., 1917, *J. Amer. Med. Ass.*, **69**, 32.  
 OTTEN, L. & KIRSCHNER, L., 1927, *Z. Hyg. InfektKr.*, **107**, 314.  
 PETRIE, G. F., 1904, *J. Path. Bact.*, **9**, 130; 1930, *J. Hyg., Camb.*, **30**, 154.  
 PETTERSSON, A., 1906, *Zbl. Bakt.*, Abt. I, Orig., **42**, 56; 1908<sup>1</sup>, *ibid.*, **45**, 235; 1908<sup>2</sup>, *ibid.*, **48**, 405; 1927, *Acta med. scand.*, **65**, 562; 1928<sup>1</sup>, *Acta Soc. Med. Suec.*, **53**; 1928<sup>2</sup>, *Z. ImmunForsch.*, **54**, 292; 1929, *Z. ImmunForsch.*, **61**, 1.  
 PETTIT, A., 1914, *Ann. Inst. Pasteur*, **28**, 663.  
 PFEIFFER, R. & ISSAEFF, 1894, *Z. Hyg. InfektKr.*, **17**, 355.  
 PFEIFFER, R. & MARX, 1898, *Z. Hyg. InfektKr.*, **27**, 272.  
 PHISALIX, C. & BERTRAND, 1895, *C.R. Acad. Sci., Paris*, **121**, 475.  
 PHISALIX, MARIE, 1922, *Animaux venimeux et Venins*, Masson, Paris.  
 PICADO, C., 1927, *C.R. Soc. Biol., Paris*, **97**, 225.  
 PIRENNE, Y., 1904, *Zbl. Bakt.*, Abt. I, Orig., **36**, 256.  
 POLETTINI, B., 1929, *Soc. internaz. microbiol. Boll. Sez. ital.*, **1**, 84.  
 PONDER, E., 1926, *J. Gen. Physiol.*, **9**, 827.  
 PRAUSNITZ, C. & MEISSNER, G., 1925, *Zbl. Bakt.*, Abt. I, Orig., **94**, 376.  
 PRITCHETT, I. W., 1925<sup>1</sup>, *J. Exp. Med.*, **41**, 195; 1925<sup>2</sup>, *ibid.*, **41**, 209.  
 RAMON, G., NOUREDDINE, O. & ERBER, B., 1928, *C.R. Soc. Biol., Paris*, **99**, 562.  
 REGENDANZ, P. & KIKUTH, W., 1927, *Zbl. Bakt.*, Abt. I, Orig., **103**, 271.  
 RETTGER, L. F. & SPERRY, J. A., 1912, *J. Med. Res.*, **26**, 55.  
 ROBERTSON, E. C., 1929, *Amer. J. Hyg.*, **9**, 75.  
 ROUX, E. & BORREL, A., 1898, *Ann. Inst. Pasteur*, **12**, 225.  
 RUBINSTEIN, P. L., 1928<sup>1</sup>, *Z. ImmunForsch.*, **55**, 107; 1928<sup>2</sup>, *ibid.*, **57**, 107.  
 SAXL, P. & DONATH, F., 1927, *Klin. Wschr.*, **6**, 1273.

SBARSKY, B., 1926, *Biochem. Z.*, **169**, 113.  
 SCHATTENFROH, A., 1897, *Arch. Hyg.*, Berl., **31**, 1; 1899, *ibid.*, **35**, 135.  
 SCHMIDT, H., 1927-8, *Z. ImmunForsch.*, **54**, 518.  
 SCHMIDT-WEYLAND, P. & KÖLTZSCH, W., 1928, *Z. Hyg. InfektKr.*, **108**, 199.  
 SCHNEIDER, R., 1908, *Arch. Hyg.*, Berl., **65**, 305; 1909, *ibid.*, **70**, 40.  
 SCHÜTZE, H., 1914, *J. Hyg.*, Camb., **14**, 201.  
 SCHÜTZE, H. & ZILVA, S. S., 1927, *J. Hyg.*, Camb., **26**, 204.  
 SCHWEINBURG, F., 1928, *Z. ImmunForsch.*, **58**, 53.  
 SEMPLE, D., 1911, *Sci. Mem. Med. Sanit. Depts. Ind.*, New Series, No. 43.  
 SICARD, J. A., PARAF, J. & WALLICH, R., 1927, *C.R. Soc. Biol.*, Paris, **97**, 217.  
 SIEGMUND, H., 1922, *Klin. Wschr.*, **1**, 2566.  
 SINGER, E., 1925, *Z. ImmunForsch.*, **43**, 285.  
 SINGER, E. & ADLER, H., 1924, *Z. ImmunForsch.*, **41**, 71, 468.  
 SMITH, T., 1913-14, *J. Med. Res.*, **29**, 291.  
 SMITH, G. H. & WASON, I. M., 1923, *J. Immunol.*, **8**, 195.  
 SNYDER, L. H., 1927, *Z. ImmunForsch.*, **49**, 464.  
 SOBERNHEIM, G. & MURATA, H., 1924, *Z. Hyg. InfektKr.*, **103**, 691.  
 STANDENATH, F., 1924, *Z. ImmunForsch.*, Tl. I, Orig., **38**, 19.  
 STEABBEN, D. B., 1925, *Brit. J. Exp. Path.*, **6**, 1; 1926, *ibid.*, **7**, 141.  
 STEWART, F. W. & PARKER, F., JR., 1926, *Amer. J. Path.*, **2**, 381.  
 STROUSE, S., 1909, *J. Exp. Med.*, **11**, 743.  
 TAYLOR, H. D. & MURPHY, J. B., 1917, *J. Exp. Med.*, **25**, 609.  
 TOPLEY, W. W. C., 1930, *J. Path. Bact.*, **33**, 339.  
 TREVES, F., 1894, *Brit. Med. J.*, i, 229.  
 TSUKAHARA, J., 1921, *Z. ImmunForsch.*, Tl. I, Orig., **32**, 410.  
 UCHIDA, Y., 1926, *Z. Hyg. InfektKr.*, **106**, 96, 275, 281.  
 WALBUM, L. E., 1921, *C.R. Soc. Biol.*, Paris, **85**, 761.  
 WAMOSCHER, L., 1927, *Z. Hyg. InfektKr.*, **107**, 240.  
 WATANABE, S., 1919, *J. Immunol.*, **4**, 77.  
 WEBSTER, L. T., 1924, *J. Exp. Med.*, **39**, 879.  
 WEBSTER, L. T. & PRITCHETT, I. W., 1924, *J. Exp. Med.*, **40**, 397.  
 WECHSBERG, F., 1902, *Z. Hyg. InfektKr.*, **39**, 171.  
 WEIL, E., 1911, *Arch. Hyg.*, Berl., **74**, 289.  
 WERKMAN, C. H., 1923, *J. Infect. Dis.*, **32**, 247.  
 WOLFF, L. K., 1927, *Z. ImmunForsch.*, **50**, 88; **54**, 188.  
 WRIGHT, A. E. & DOUGLAS, S. R., 1903, *Proc. Roy. Soc.*, **72**, 358; 1904, *ibid.*, **73**, 128; **74**, 147, 159.  
 WRIGHT, A. E., COLEBROOK, L. & STORER, E. J., 1923, *Lancet*, Lond., i, 365, 417, 473.  
 WRIGHT, H. D., 1927, *J. Path. Bact.*, **30**, 185.  
 WYSSOKOWITSCH, W., 1886, *Z. Hyg.*, **1**, 3.  
 ZILVA, S. S., 1919, *Biochem. J.*, **13**, 172.

## CHAPTER III. THE PRODUCTION OF ACTIVE IMMUNITY TO BACTERIAL AND VIRUS INFECTIONS OF MAN AND ANIMALS.

BY J. C. G. LEDINGHAM AND H. L. SCHÜTZE (LISTER INSTITUTE, LONDON).

### Introduction.

IT is proposed to devote this chapter, as strictly as the subject matter will allow, to the presentation of evidence showing the extent to which a solid immunity against infection is capable of being conferred by deliberate introduction into the system of the causative agent living or dead. At first sight the injection of the living organism might be expected to defeat the object aimed at, were it not borne in mind that the results of experimental infection, even with the most virulent organisms, are largely dependent on the dose and route of introduction and on the natural resistance of the inoculated subject. Experimental infection also has to be effected for the most part by injection of laboratory cultures of the causative organism, or, in the case of ultramicroscopic viruses hitherto not artificially cultivated, by injection of infective tissues and exudates. By taking advantage of the wide variations in pathogenicity exhibited by such materials and of the further modification of such pathogenicity occasioned by route of introduction, it is possible to induce even with the living virulent organism a type of disease so modified as to be unimportant but yet capable of conferring solid immunity against the naturally acquired infection.

Historically the use of the living virulent organism as the immunizing agent preceded that of the 'attenuated' living variant. Variolation, for example, owed what success attended it to the fact that the virus was applied to the skin or an external mucous surface where local defences tended at least to restrict generalization with the risk of fatal issue. In more recent times the use of living cultures of typhoid or cholera germs was rendered possible by taking advantage of their relative innocuousness to man when introduced into the subcutaneous tissues. With the living virulent organism as the immunizing agent modern systems of active immunization, whether of man or animals, have now little or no concern owing to the dangers involved in the immunization process itself. The principle of the 'attenuated' virus, on the other hand, at first empirically employed by Jenner, became the foundation stone of Pasteur's experimental efforts to secure immunity to bacterial and virus diseases of man and animals. So far as human diseases are concerned the use of the attenuated living virus is now restricted to two procedures only, viz. vaccination against smallpox by aid of vaccinia, and post-infectious immunization against rabies by one or two still current procedures, which, however, are being rapidly replaced by the use of killed virus (see below).

## 78 PRODUCTION OF ACTIVE IMMUNITY TO BACTERIAL

To these two may be added the employment of the Calmette-Guérin strain of *B. tuberculosis* (B.C.G.) for conferring immunity against tuberculosis, though this effort is still in the experimental stage and its assessment remains a matter of controversy.

In human bacterial diseases of epidemic importance, e.g. typhoid, paratyphoid, cholera, plague, dysentery, &c., the efforts of the last thirty years have been directed mainly to the securing of immunity against the naturally acquired disease by the use of the killed organism as immunizing agent and with the results achieved in this direction we shall be largely concerned in this chapter. The risk that may be involved in depending too rigidly on the 'fixed' character of an attenuated variant and the acknowledged undesirability of employing the living organism in man, even by an innocuous route, has rendered necessary the study of the immunizing powers of 'killed' antigen; and though a fair measure of success has been achieved, it cannot be said that the degree and duration of the immunity conferred by the dead antigen at all approach that conferred either by the living 'attenuated' organism which is given a chance to multiply, or by recovery from the natural infection. Recent studies of bacterial variation, however, more especially with regard to the chemical structure and immunizing properties of bacterial antigen, have explained many former obscurities and have placed the problem at issue on a more secure basis. A later section (p. 98) will be devoted to a consideration of these newer developments which have greatly clarified the situation, more especially perhaps by the light they have thrown on the relationship between nature and quantity of antibodies produced and the experimentally ascertained immunity conferred by the antigen.

While the employment of the 'attenuated' virus in preventing human disease is now confined to the few instances mentioned above, in animal diseases the principle of the 'attenuated' virus still holds the field, though in most of them attempt has been made to reduce the risk of fatalities in course of immunization by simultaneous injection of virus and the corresponding antiviral serum. In illustrating the various types of immunization, detailed reference will be made only to those diseases of man and animals in which the experimental evidence of efficacy, or the field evidence, or both, are sufficiently cogent to merit serious consideration.

### Methods of Immunization which have been Employed at Various Periods and Criteria of Assessment.

#### THE PRE-JENNERIAN AND JENNERIAN PERIODS. VARIOLATION AND VACCINATION.

Variolation of man with human smallpox material and vaccination of man with cowpox matter were both claimed to produce comparatively mild diseases, recovery from which gave immunity to naturally contracted smallpox. In the former method the risk of generalization with possibly a fatal issue could not with certainty be controlled nor could the normally

contagious properties of the virus be kept in complete check. In the latter method, on the other hand, risks occurring in the process of immunization were regarded as negligible, the cowpox virus being believed to possess a fixed but low degree of virulence for man. That in both cases the route of introduction of the virus was of importance in determining the mitigation of clinical symptoms was clearly understood by exponents of variolation and vaccination of these times. Thus Jenner (1798), writing of variolation and its dangers, writes : ' I have known an inoculator whose practice was to go deep enough, to use his own expression, to see a bit of fat and there to lodge the matter ; the great number of bad cases and the fatality which attended this practice was almost inconceivable, for let it be recollected *that it is only from a different mode of receiving the infectious particles* that the difference between inoculation (i.e. variolation) and the natural smallpox arises '. With Jenner's views as to the natural history of cowpox and smallpox we are now little concerned, though it was a subject which obsessed him greatly. To him the common link between human smallpox and cowpox was the disease of the foot of the horse known as ' grease '. According to Jenner, this disease when propagated through man became the highly virulent disease known as smallpox. When propagated through the cow, on the other hand, it became the relatively mild disease known as cowpox. ' It is curious ', he writes, ' to observe this matter (from the horse) which acquires new properties by passing from the horse through another medium, that of the cow ; not only is its activity hereby increased but those specific properties become invariable which induce in the human institution symptoms similar to those of the variolous fever and effect in it that peculiar change which for ever renders it unsusceptible of the variolous contagion '. When Jenner found that horse grease itself did not protect against smallpox he concluded that only after passage through the cow must it acquire the property of affording protection against human smallpox. These citations show that Jenner was fully alive to the variation in the properties of a disease or virus which might ensue on passage through an animal not its proper host, though it was left for Pasteur to exploit this principle more completely. One other citation from Jenner's writings is of interest in that it concerns a point which is still a matter of active enquiry. He says ' Although the cowpox shields the constitution from the smallpox and the smallpox proves a protection against its own future poison, yet it appears that the human body is again and again susceptible of the infectious matter of the cowpox '. It is evident that problems which still exercise students of vaccinia and virus diseases generally were clearly anticipated by Jenner and other writers of his times (e.g. Pearson and Woodville).

The principles and practice of vaccination against smallpox are dealt with elsewhere (Vol. VII, Chapter VII). Statistical evidence of its value in protecting the vaccinated from attack and in moderating the mortality rate cannot be discussed here. Experimental evidence of its power to

protect against smallpox is amply afforded by the failure of smallpox virus to produce its characteristic exanthem in the tissues of vaccinated men and monkeys after deliberate variolization.

To the period we are discussing belongs also the ancient practice of ovination, a method of controlling sheep-pox on lines precisely similar to variolization (see Hutyra and Marek, 1910). Moreover, before Pasteur's entry into this field, methods of vaccinating cattle against pleuro-pneumonia by insertion of virulent lymph at the root of the tail had been extensively and successfully practised (Willems, 1852).

#### THE CONTRIBUTIONS OF PASTEUR. EXPLOITATION OF 'ATTENUATED' VIRUS (FOWL CHOLERA, ANTHRAX, SWINE ERYSIPELAS, RABIES).

In so far as Pasteur concerned himself with the problem of active immunization against infectious disease by aid of the living 'attenuated' virus, he followed in the footsteps of Jenner. He embarked on this work during the last few years of his working life and while he was approaching his sixtieth year. The era of 'pure cultures' and precise quantitative bacteriological technique had scarcely dawned. A disease of economic importance had only to be brought to his notice to stimulate him to discover to his own satisfaction its bacterial origin, if not already known, and then forthwith to attenuate it with a view to its employment in prophylaxis. The papers in which Pasteur recorded his contributions to this subject do not supply those details now demanded of the modern bacteriologist, and it is, therefore, all the more remarkable that with the tools at his disposal he was able to institute methods which have stood the test of time. He had his set-backs with the 'impure culture' trouble, and Koch was not slow to point out the danger of relying too much on the fixity of the attenuation. On the other hand, his young contemporary admitted frankly the fundamental importance of Pasteur's success in attenuating the virulence of an organism like *B. anthracis*. This work alone makes Pasteur the pioneer worker in the field of bacterial variation as now understood.

Here we propose to set out very briefly the chief features of his work on active immunization without entering into any discussion of points that are doubtful in the light of modern knowledge. Some of the claims Pasteur made would seem to remain practically as he stated them, the ground not having been retilled since.

*Fowl cholera* (Pasteur, 1880<sup>1</sup>). The causative organism recovered from sick fowls he regarded as similar to that figured by Perroncito in 1878 or by Toussaint in 1879. Its relative pathogenicity he investigated for rabbits, fowls and guinea-pigs and he decided to attempt attenuation by altering the conditions of growth. This he appears to have done simply by repeated subculture at intervals in broth exposed to air so that the organisms were well oxygenated.

Little or no attenuation in virulence occurred when the broth-tubes were sealed up and only a little air left above the broth. Noting that the

protection conferred by the most attenuated strain increased with the number of inoculations, he fancied that, as in the test-tube, so in the animal body, the available pabulum for the attenuated strain must be first exhausted before the body becomes 'immune' or unable to support a fresh population. One inoculation of the attenuated strain cannot, he said, complete the business because the bacterium has to compete with the body-cells for oxygen and dies out before it exhausts the fluids of the animal. Two or more inoculations, therefore, are needed.

*Anthrax* (Pasteur *et al.*, 1880, 1881<sup>1</sup>; Pasteur, 1880<sup>2</sup>, 1882, 1883). Pasteur and his pupils, Chamberland and Roux, had been concerned with the ætiology of anthrax for a few years before an attempt was made to produce immunity by the inoculation of a weakly pathogenic strain of the organism. Toussaint (1880) had, indeed, demonstrated some degree of protection conferred on sheep by inoculation with the heated or phenolized serum of a sheep dead of anthrax. Pasteur's experience with fowl cholera led him to attempt attenuation of *B. anthracis*, and this he secured by growth on artificial media at a temperature of 42° to 43° C. After 8 days' growth the organism was found to be non-virulent for guinea-pigs, rabbits and sheep. Grown for 12 days it was non-virulent for adult guinea-pigs; after 31 days it was able to kill young mice only; after 43 days even mice were not killed.

The famous field experiment at the farm of Pouilly-le-Fort, near Melun, took place in May 1881. Twenty-four sheep, 1 goat and 6 cattle were given two doses of vaccine of unequal attenuation on 5th May and 17th May, and on 31st May the test-dose of virulent anthrax was given to these and to an approximately equal number of unvaccinated controls, viz. 24 sheep, 1 goat and 4 cattle. Of the vaccinated animals only one sheep was reported dead on 4th June. This animal was pregnant, and contained a foetus *in utero* which had been dead for some time. All the unvaccinated sheep and the goat died of anthrax, while the four unvaccinated cattle had severe local reactions but survived.

The experiment of Pouilly-le-Fort furnished conclusive evidence that the inoculation of an attenuated *B. anthracis* could confer protection against infection with a virulent variety in an animal (sheep) naturally susceptible to the disease. The fact that in more recent times the Pasteurian anthrax vaccines for field use have been largely replaced either by mixtures of the living organism and immune serum or by glycerinated suspensions of spores derived from attenuated cultures, is due to the difficulty unrealized by Pasteur of securing stability and consequently complete innocuousness of the attenuated culture.

*Swine erysipelas* (Pasteur *et al.*, 1882<sup>2</sup>; Pasteur and Thuillier, 1883). In March 1882, Thuillier, a pupil of Pasteur, was deputed to study swine erysipelas (*rouget du porc*) in the Vienne province. Apparently he succeeded almost at once (15th March, 1882, according to Pasteur) in isolating a likely causative organism which Pasteur and he satisfied themselves was the *vera causa* of this disease. 'Notre premier soin fut

## 82 PRODUCTION OF ACTIVE IMMUNITY TO BACTERIAL

de chercher ensuite à atténuer la virulence du microbe', and here difficulties arose owing to the fact that the native races of swine showed differences in susceptibility to rouget. The method of attenuation employed in this case was entirely novel. When the organism of swine erysipelas was inoculated intramuscularly into pigeons, they died in 6 to 8 days. Further passage through pigeons increased the virulence both for the pigeon and the pig. Passage through rabbits had the opposite effect, the strain becoming much less virulent for the pig. Rabbit passage consequently afforded the attenuation desired for prophylaxis in the field.

*Rabies* (Pasteur *et al.*, 1881<sup>2</sup>, 1882<sup>1</sup>, 1884 ; Pasteur, 1885, 1886). In 1881 Pasteur, Chamberland, Roux and Thuillier reported that the seat of infection in rabies was the brain, in proof of which statement they had successfully produced rabies in dogs by inoculating them subdurally with brain material from a mad dog. In 1884 came the report that passage of the virus through rabbits, guinea-pigs and monkeys caused the virus to acquire a 'fixed' degree of virulence for each species when introduced intracerebrally. The most remarkable effect of the passage, however, was the change in the character of the virus, which was now found incapable of infecting dogs, rabbits and guinea-pigs by the subcutaneous route. The change in infectivity suggested to Pasteur the possibility of employing this fixed virus for the post-infectious immunization of man. The original method (1886) involved the use of rabbit cords dried for varying periods. A commencement was made with cords dried for 8 to 12 days which were avirulent for the rabbits. This was followed by inoculation of cords dried for shorter and shorter periods, and which retained their virulence for the rabbit.

The method is still in use in certain countries, but in others has been replaced either by gradually increasing doses of the fixed virus or by carbolized or formolized virus (see below, p. 95).

### MODIFICATIONS OR AMPLIFICATIONS OF THE PASTEURIAN METHOD WHICH ARE STILL CURRENT IN VETERINARY DISEASE CONTROL. ALSO SOME APPLICATIONS, NO LONGER CURRENT, TO BACTERIAL DISEASES OF MAN (TYPHOID, CHOLERA, PLAGUE).

Though Pasteurian principles of active immunization continue to find their chief field of exploitation in veterinary diseases whether of bacterial or of virus origin, considerable departures have been made in most cases from the classical tradition. The main departure has been the addition of a quantum of the specific immune serum to the immunizing dose of living virus or bacterium with the object of reducing immunization casualties to a minimum and of completing the process in one instead of two or more stages. The addition of the specific serum is calculated to confer some degree of passive immunity which permits the contraction of the disease in a mild form and consequently the securing of some active immunity therefrom. The types of immunizing material which have been

employed in the prophylactic control of various veterinary diseases are exceedingly numerous and their exact composition is not always readily ascertained owing to the fact that they emanate for the most part from commercial firms whose veterinary biological products are not under state control as those for human use now are. Nothing in the way of standardization of such products has yet been attempted. Some account of these products is given in the chapters dealing with the various veterinary diseases and to these the reader is referred. Here it seems appropriate to consider some general features of the problem of veterinary disease control by prophylactic immunization and the difficulties that surround it. Recently this problem has been very fairly discussed by Schnürer (1927), who, while admitting freely that in certain diseases such as swine fever, swine erysipelas, anthrax and canine rabies control by active immunization methods has secured an assured position, concludes that the best hope for the future lies in the improvement of stock-breeding and all that this implies. Though a method of prophylaxis may be proved effective, it may not be expedient to exploit it if it involves a risk of disseminating infection by carriers or if the disease is not too prevalent to render its control by slaughter possible. For example, even where immunization is practised as for canine rabies, it may be wise to slaughter immunized dogs that have been bitten by rabid dogs. Such practice would naturally defeat statistical treatment of results, but for various reasons it is even more difficult in veterinary than in human disease to secure data that a statistician would accept for analytic purposes. In the first place, vaccines recommended for the control of the same infection may differ widely in composition and potency; the identification of immunized animals has not been entirely solved by the various branding devices in use, and even if these were satisfactory the frequent buying and selling and disposal by slaughter, perhaps at remote markets, preclude the collection of accurate figures which might determine the value of the method in the field. Frequently, also, no animals are left uninoculated, so that estimates of efficiency are based on comparisons of past and present experience—a notoriously fallacious form of reasoning. Schnürer points out that where compensation is given by the purveyor of the vaccine for loss of animals through or in spite of the vaccine, the figures are liable to be far more accurate. In the case of swine erysipelas, we are told that the original guarantee was protection for one year, but experience reduced this figure to five months, which may be reckoned, therefore, the mean duration of the immunity conferred.

It may in the future be considered good policy to institute some state control over commercial veterinary preparations for prophylactic use or to insist, at least, that tests of efficiency be carried out in small experimental series adequately controlled. Determinations of efficiency derived from experiments on small animals not susceptible in nature to the disease in question afford valuable presumptive information, but the small controlled field experiment remains the crucial test.

## 84 PRODUCTION OF ACTIVE IMMUNITY TO BACTERIAL

*The use of living vaccines in the control of human disease.* Efforts to produce active immunity in man by aid of living bacterial vaccines are now largely matters of historical interest, but they demand some notice in view of the fact that they reflect the well-founded belief of the time that, if technically practicable and safe, the application to man of Pasteurian methods was likely to yield the higher grades of immunity. The first attempt of the kind was that of Haffkine (1892), who prepared two living cholera vaccines I and II, the former being an attenuated culture obtained by growth at 39° C. with much aeration, and the latter a strain whose virulence was exalted by repeated guinea-pig passage. The history of this form of vaccination and the results of its employment in India (1894-6) are summarized by Haffkine in a retrospect of the subject published in 1913 (Haffkine, 1913). In his later work the first or attenuated culture was omitted and only the virulent vaccine No. II employed. Hetsch (1904) also recommended the omission of the first vaccine. The virulent organism, he found, was immediately killed in the subcutaneous tissues and yielded better protection than the avirulent strains. Nicolle's (Nicolle *et al.*, 1912) attempts to immunize man by living vibrios intravenously injected were not followed up for very obvious reasons.

Similarly, living typhoid vaccines have been employed in the past by individual workers in small-scale series (Castellani, 1913; Broughton-Alcock, 1912, &c.). These attempts were based largely on the failure of Metchnikoff and Besredka (1911, 1912) to immunize chimpanzees with killed typhoid vaccine and their success with the living organism sensitized with antiserum. The test-dose was given *per os*.

Living attenuated cultures have even been used for the immunization of man against plague. Thus Strong (1906) inoculated intramuscularly 42 persons with living attenuated *B. pestis*, experiments on monkeys having shown that such cultures die out in the subcutaneous tissues within 24 hours. The fact that guinea-pigs, rats and monkeys immunized with living avirulent *B. pestis* acquire a much higher immunity than when killed agar or broth cultures are used was shown clearly by various workers at an early date (Kolle and Otto, 1903; Strong, 1907, &c.).

### POST-PASTEURIAN PERIOD. INTRODUCTION OF THE 'KILLED' ANTIGEN AS THE IMMUNIZING AGENT. APPLICATION TO TYPHOID, CHOLERA, PLAGUE, &c., AND CERTAIN VIRUS-DISEASES OF ANIMALS.

We have seen that the principle of the attenuated virus has deservedly continued to occupy a premier place in the control, by active immunization, of animal plagues, whether of bacterial or of virus origin. It is unfortunate on scientific grounds that the data on which the reputed efficiency of these methods rests are statistically so unsatisfactory, but there is this to be said that in all probability the scope and application of the attenuated virus for immunization purposes would not have been so widespread and persistent were there not behind it the faith that recovery from the

minor disease induced by the attenuated virus must inevitably confer immunity against the naturally acquired infection. Though some few writers have expressed the view that killed antigen administered in sufficient amount can satisfactorily replace the living, opinion is now fairly general that the degree and duration of the immunity conferred by the attenuated virus cannot be approached by any method which substitutes the dead antigen for the living bacterial cell with its capacity to multiply. The superiority of the living antigen in these respects may not be due simply to the more intense and more widespread stimulation of susceptible cell tracts by the living multiplying invader. Evidence is available (see p. 87) that the immunity produced in response to the living organism may differ in quality from that elicited by the killed antigen, and possibly, also, the permanent change in the reactivity of the susceptible cells induced by the living virus may be of different quality from that brought about by the dead antigen. A further possibility has recently been suggested which may indeed prove of general application, though at present the relative data are scanty, viz. that long-continued immunity accruing from deliberate or natural infection with the living organism may be due in some measure to the continued presence of the virus in the body after recovery. When the virus finally leaves the body the immunity would lapse. There is now, however, abundant evidence from many sources that, even if the immunity lapse, whether that immunity follows the introduction of living or of killed antigen, minimal quantities only of additional antigen are required to stimulate the antibody-forming mechanism and to restore the immune state.

#### *Study of the killed antigen.*

The period immediately following the death of Pasteur (in 1895) witnessed the introduction of a new principle of immunization dictated by the necessity of avoiding in man the dangers lurking in the attenuated living virus as the immunizing agent. The achievements of the era we now propose to discuss are concerned almost solely with efforts to control epidemic diseases of man by less risky methods of immunization. The immediate impetus to this advance came from Haffkine's successful attempts to control cholera on lines that at least imitated Pasteurian principles.

*Criteria by which the possible efficacy of a killed antigen for immunization of man may be judged.* The best evidence of production of solid immunity in response to a killed vaccine is derived necessarily from experiments in which animals naturally susceptible to the particular infection are immunized and at some period following the close of immunization are given such dose of the living virulent organism as will inevitably kill the unimmunized controls or produce in them a characteristic disease syndrome. Such a method of test must of necessity be restricted to animals. In man one must as a rule be content with the introduction of a selected antigen and then await its evaluation as an efficient or inefficient immunizer by

statistical analysis of epidemic data. It is certainly a debatable point how far the data obtained by immunization of laboratory animals such as guinea-pigs with typhoid or cholera antigen can be used for estimating the probable value of the same antigens in man, particularly in view of the fact that the parenteral inoculation of these experimental animals with adequate doses of living virulent *B. typhosus* or *V. cholerae* produces an acutely fatal infection unaccompanied by clinical features characteristic of the disease in man. We do not share the view that such data have a very limited bearing on the prevention of human disease by immunization with antigens so tested. Physiological responses to antigens are, on the whole, remarkably uniform in the mammalian series and we have no reason to suppose that the protection afforded by a particular antigen against an otherwise acutely fatal experimental infection in an animal lacks its counterpart in the human subject similarly immunized and exposed to the natural infection in the field.

Another method of animal experimentation which undoubtedly approaches more closely to nature is that employed by Topley and his colleagues in mouse populations, the principle employed being to immunize series of mice with particular antigens and then to deposit them amid surroundings in which they are liable to contract the infection against which protection is sought. The data so obtained have a precision which no statistical data of human epidemic infections can approach.

#### *Antibody-production as criterion of efficacy of vaccine.*

Throughout the period we are considering, in fact until quite recent years, the efficiency of an antigen as an immunizing agent has been supposed to be a direct function of its antigenic or antibody-stimulating power. The work of Pfeiffer had revealed the presence of lytic substances in the sera of convalescents from typhoid fever and of animals inoculated with typhoid antigen. It was natural to suppose that the inoculation of man with typhoid antigen would equally be followed by the appearance of lytic substances in the serum, demonstrable by test-tube methods or by passive protection in an experimental animal. The fact that these antibodies played an important role in determining the issue of an experimental infection and the subsequent demonstration that the serum of immunized animals contained, in addition to lysins, tropins and opsonic amboceptors capable of sensitizing organisms preparatory to their phagocytosis by leucocytes, made it highly probable that the degree of protection following the parenteral introduction of an antigen would be directly proportional to the antibody-titre attained in the course of immunization.

Opinions differed as to the relative importance of lysin-, opsonin-, and agglutinin-development as evidence of antigen efficiency in this sense. While some have stressed the importance of lysins, others have laid more emphasis on opsonin development, and, as we shall see, many weighty inferences have been drawn from agglutinin titres following inoculation with different antigens, although the functional role of agglutinins *in vivo*

was held to be a very subordinate one. The employment of agglutinin-titres as criteria of efficacy had this merit, however, that their estimation was—as a rule simple and straightforward. It is perhaps strange, in the light of more recent knowledge, that the mere development of antibodies—a purely physiological response to antigen—should have been so long regarded as synonymous or practically synonymous with a state of protection lasting it might be for an undetermined period, when it was realized that, by the time exposure to experimental or natural infection occurred, a previously high antibody titre might have fallen to normal levels. In the immunization of man this natural fall in antibody-titre precluded any very reliable correlation between a previously demonstrated titre and a susceptibility or insusceptibility to the natural infection. At the most it was possible to effect some sort of correlation with the number of inoculations given and the period elapsing between the last inoculation and the contraction of the natural infection. As a matter of fact we are still inadequately informed as to the precise correlation between antibody titre and resistance to experimental infection performed while that titre is at its height. Similar difficulties have been met in attempts to correlate the protective action of a serum with its antibody-content, e.g. agglutinin-content. The literature on this subject is very contradictory, and on the whole there would appear to be little evidence of any direct relationship between the strength of agglutinins and the outcome of an experimental infection or the value of the serum in terms of passive protection. We may cite, e.g., the experiments of Pratt-Johnson (1921), who attempted to immunize rabbits against a highly virulent strain of *B. suispestifer* by three different antigens—(a) a killed vaccine of the homologous strain of *B. suispestifer*, (b) a killed vaccine of an allied organism (*B. aertrycke*), and (c) a living vaccine of this latter. No protection was afforded by either of the killed antigens, but the animals immunized with the living non-virulent allied organism (*B. aertrycke*) were very definitely protected, and that too in spite of the fact that their sera showed only a very feeble agglutinin-development for the organism to which they proved resistant. Similarly, in the course of a study by Enlows and Brooks (1928) of the comparative agglutinin- and antitoxin-content of antidiarrhoeal serum the conclusion is reached that no parallelism can be detected. On the other hand, as these writers point out, it is impossible to dispute the evidence 'as to the efficiency index of anti-serums secured from the protection of certain animals against lethal doses of the specific living organisms, and this despite the fact that at times no definite antitoxin, agglutinin nor bacteriolysin may be demonstrated'.

That many of the difficulties and discordances encountered in this question of relationship or lack of relationship between antibody-titre estimated *in vitro* and solid immunity or passive protective value of serum are likely to be solved or clarified by the newer work on structure of bacterial antigen will be shown later, vide p. 98. Here we may simply note that this recent work has demonstrated not only the complexity of

## 88 PRODUCTION OF ACTIVE IMMUNITY TO BACTERIAL

the antigen in bacterial cells but the partition and diversity of the antigen in particular variants of the same race. Some variants, for example, possess a diversity of antigens, only one of which would appear to be antigenically efficient in producing solid immunity. Another variant of the same organism may contain no antigen of this order whatever, so that immunization with it is not followed by conferment of solid immunity against the living virulent organism. These various antigens have their corresponding antibodies, and it now remains to be investigated how far the special antibodies that are elaborated in response to the antigen that confers solid immunity can be correlated qualitatively or quantitatively with the resultant protective state. The separation of one antigenic constituent enables a finer differentiation of the antibodies produced by it from those produced by other antigen fractions or by the unaltered bacterial protoplasm. If we accept the view that the injection of any *pure* antigen produces as a rule a single sensitizing body which may be demonstrated by a variety of test-tube techniques, it may eventually be proved that striking variations in the amount and time of appearance of the sensitizing body as tested by various techniques may really be due to the fact that antibody development is taking place independently in response to the component fractions of the unaltered antigen injected. When it is found that a particular antigenic constituent is capable of conferring solid immunity, study of the antibodies formed in response to this constituent may enable us to associate the presence in the serum of a particular antibody with a state of probable protection. The method employed to demonstrate this antibody *in vitro* may be immaterial. The fact, however, that antibodies may in time fail to be detected *in vitro* while immunity or passive protective effects by serum may persist, has the effect of reducing this whole problem of active immunity to one of altered cell-reactivity. The evidence in favour of this altered reactivity is well established and dates back to the observation by Rufus Cole (1904) that an animal which has developed a certain antibody-content as the result of immunization and has then been allowed to lie fallow until the antibodies have fallen to minimal levels, will respond to the inoculation of a fresh dose of antigen in a manner and degree entirely different from the normal or unimmunized animal. Active immunity may, in fact, reside not in any antibody-content demonstrable pre-infectionally, but in the reactive capacity of those cells of the reticulo-endothelial system which have been trained to respond quickly to the presence of antigen by mobilization or fresh manufacture of antibody destined to combat infection. In what follows we propose to summarize briefly, in the light of the foregoing considerations, what has so far been achieved with killed vaccines, confining ourselves strictly, however, to those diseases of man and animals in which the available evidence is of sufficient interest and cogency. Only those features of the various immunization schemes will be discussed which are of general interest and which illuminate the subject of active immunization as a whole.

*Typhoid and cholera.*

In this country the work of Wright laid the foundation of antityphoid vaccination as a practical measure of disease prevention in the field. With his work must be coupled that of Leishman and his collaborators, whose investigations were conducted under the auspices of the Antityphoid Committee which was appointed in 1904 and reported in 1912. Though the criterion of probable protective value adopted by these workers, viz. the antibody-stimulating property of the vaccine, was, in the light of present knowledge, misleading, it is important to note some of the results they obtained and the inferences they drew therefrom. The type of vaccine with which they started operations was one made from a culture of low virulence heated for 15 minutes at 62° C., and the doses decided upon as the result of antibody titration in human volunteers and the local reactions encountered were roughly 500 millions followed by 1,000 millions.

*Effect of heat on vaccine.* From rabbit tests it was decided on the strength of agglutinin-titres and phagocytic indices that the most efficient vaccine was that which had been heated (killed) at 53° C., the chloroformed vaccines coming next and the vaccine heated at 60° C. last. The differences, however, between the antibody titres were very small and scarcely sufficient to justify the inference drawn.

*Age of vaccine and immunizing response.* The Committee tested as to antibody production and phagocytic values three vaccines, one freshly prepared, one three months old, and a third, six months old. Between the two former there was little to choose, whereas the third had practically no antibody stimulating power. The result, which would seem not very readily explicable, led to the decision to employ no vaccine older than three months.

*Virulence of strain and immunizing power.* An important pronouncement was made as the result of experiments designed to determine whether a recently isolated virulent strain of *B. typhosus* or an old non-virulent laboratory strain was the better suited for vaccine preparation. No significant differences in agglutinin and opsonin development were detected, and it was decided that the virulence or non-virulence of the strain was immaterial. The inference thus drawn was almost certainly, in the light of recent knowledge, unjustified for reasons that will be discussed in the next section. At the period in question, though there was a general belief that the possession of virulence by an organism enhanced the protective value of the vaccine prepared from it, definite experimental proof of the superiority of the virulent strain in this respect was not available except perhaps in the case of *B. pestis*.

Inferences were drawn from certain serological differences between virulent and avirulent races. Thus, Pfeiffer and Kolle (1896) reported that a virulent *B. typhosus* required far more immune serum to lyse it in the guinea-pig's peritoneum than one of low virulence. Wassermann (1903), on the other hand, did not find any parallelism between antibody-production and virulence in *B. typhosus*.

## 90 PRODUCTION OF ACTIVE IMMUNITY TO BACTERIAL

In the case of the cholera vibrio, Strong (1904) found that injection of the virulent living organism or its autolysate yielded in rabbits a slightly higher titre of agglutinins and bactericidins than the living avirulent organism—a result in agreement with experiments of Pfeiffer and Friedberger (1902). The latter concluded that virulent strains absorb agglutinins and lysins from an immune serum more readily than avirulent strains. There was, however, no settled opinion on the point, and Meinecke, Jaffé and Flemming (1906) reported, in fact, that the binding power of a strain was independent of its virulence. The Antityphoid Committee had contemplated an investigation of the protective properties of vaccines by guinea-pig experiment and it is unfortunate that difficulties met with in the course of their preliminary trials of this method—variations in individual resistance, maintenance of virulence of test strains, &c.—led them to abandon the method. Such difficulties are real but they can be overcome by employing sufficiently large series of animals to enable statistical evaluation of results. The experiments of Weber (1916, 1917) on guinea-pigs represent an important effort to secure a direct estimate of the value of typhoid and cholera antigens for prophylactic purposes. The chief points that emerged were that protective action varied directly with dose of antigen and that the immunity appeared to develop slowly. With regard to this latter point he states that of 54 animals tested 2 to 4 weeks after the immunizing dose was given, 46 per cent. survived, while of 68 tested at 5 to 7 weeks, 82 per cent. survived. It will be admitted, however, that the duration of the immunity conferred by killed antigen has not been adequately investigated by experimental methods and that further research is called for. In the great majority of experiments in which the protective value of a vaccine is being tested, it has been customary to choose some period for the test not later than ten days or a fortnight after the end of the immunization process. We reserve for the following section a discussion of some results of recent experimental work with typhoid antigen which bear chiefly on the importance of virulence or O-antigen-content of the strain employed for immunization.

*Brief summary of statistical analysis of results of antityphoid and anti-cholera vaccination in the field.* Greenwood and Yule (1915) lay down certain criteria which the data must satisfy if valid statistical inferences are to be drawn. We may enumerate these conditions here though the reader is advised to consult the original for a full discussion of their implications.

- (1) The persons must be *in all material respects alike*.
- (2) The effective exposure to the disease must be identical in the case of inoculated and uninoculated subjects.
- (3) The criteria of the fact of inoculation and of the fact of the disease having occurred must be independent.
- (4) The number of observations must be sufficient.

When these conditions are satisfied Greenwood and Yule apply to the data the  $\chi^2$  criterion of Pearson, the calculated value of which supplies



the probability that differences in incidence or fatality rates signify an organic distinction between the inoculated and uninoculated classes. The higher the value of  $\chi^2$  the smaller the probability that the differences observed are due to random sampling. The only data on typhoid vaccination amenable to statistical manipulation and then available were those concerned with British troops in India (see Report of Antityphoid Committee). Analysis of these data led the authors to conclude that 'the case in favour of antityphoid vaccination as a practical measure was very strong'.

Various considerations into which we cannot here enter militate against the application of strict statistical analyses to the vast data collected during the war, but three of these difficulties may be noted, viz. (1) the vast preponderance of inoculated over uninoculated persons, (2) the overlapping of inoculation and exposure to infection, and (3) difficulties in determining with accuracy the scope of the attacked class owing to the employment, in a considerable proportion of cases, of methods of diagnosis the validity of which is disputed.

Elsewhere (Vol. IV, pp. 50 to 58) are recorded the analysis of the British enteric data of the war period and the interpretations put upon them. Though, as we have said, the data are not amenable to accurate statistical analysis, there can be little doubt that they afford strong presumptive evidence of the value of anti-enteric inoculation. There would appear to be general agreement, however, that further investigation of the subject on experimental lines dictated by recently acquired knowledge of bacterial antigen (see p. 98) may lead to improvement in field methods. Friedberger's (1919) somewhat extreme views with regard to the uselessness of killed antigen for prophylactic purposes have given rise to much controversy. They cannot be usefully discussed here. Some considerable support for these views has come from Miyashita's (1929) observations on the results of anti-enteric inoculation in Japan. He concludes that case-mortality is not less in inoculated than in uninoculated and that the disease does not run a milder course in the vaccinated. That vaccination mitigates the attack when it does not prevent it is strongly held both in this country and in Germany, but the view is not unchallenged. Much has been made, particularly in Germany, of the fact that since the war the incidence of enteric has been very much higher in women than in men, and the phenomenon has been attributed to persistence of immunity among adults from vaccination during the war. Friedberger (1927) also challenges this point and refers to the fact that during the war the immunity resulting from inoculation was not believed to last more than twelve months. His opinion is that if the facts are as stated, the phenomenon is more likely to be due to actual parasitization of adults during the war by the living organism and to sub-infections for which there was ample opportunity, rather than to deliberate inoculation with typhoid antigen. Typhoid vaccine alone was used in the German Army. Yet we are informed by Weigmann (1926) that in

Schleswig-Holstein there is a similar preponderance of paratyphoid fever among adult women. It is greatly to be hoped that experimental epidemiology, as its scope enlarges, may give the key to problems that field data in man cannot at present unequivocally supply.

*Statistical evaluation of anticholera vaccination.* Reference may be made to Greenwood and Yule's (*loc. cit.*) analysis of the data recorded by Haffkine (see his Monograph of 1913) and Powell (1896) in India following the use of living cholera vaccine, by Murata (1904) in Japan, by Nijland (1911, 1913) in the Dutch East Indies, and by Savas (1914) in the Balkans (2nd Balkan War). Greenwood and Yule conclude that Haffkine's data with reference to the Cachar Estate coolies (1895-6) yielded a value of  $\chi^2$  such that the difference of incidence in inoculated and uninoculated could not be attributed to random sampling. The data of Nijland also yielded results favourable to vaccination, while in one section only of the data of Savas, *viz.* that dealing with the experience of the Sanitary Corps, could it be fairly definitely decided that inoculation was responsible for the observed results. This particular section of the Greek Army had been vaccinated fully against cholera *before the disease broke out*. A recent statistical analysis by Russell (1927) of the results of anticholera vaccination in Madras Presidency also affords clear evidence of the value of the method. The author set out to make a comparative trial of cholera bilivaccine (oral) and the ordinary cholera vaccine (subcutaneous) and concluded that both conferred a high degree of immunity though the latter was in his opinion superior. He was able to show, for example, that the immunity present five days after a single dose of cholera vaccine (subcutaneous) was about as high as that present at a period of three days after a full course of the oral vaccine.

### *Plague.*

We have already noticed some results achieved with living attenuated plague cultures as prophylactic antigens. In the attempt to improve the protective value of plague vaccine for human use, renewed attention has been given in recent years, particularly in India, to experimental trials on rats and guinea-pigs of plague antigen prepared in various ways. To some of these we shall now allude.

*Virulence of strain.* That a highly virulent strain is a necessary preliminary in the production of such vaccine is now generally agreed. Naidu, Malone and Avari (1926), for example, increased the protective power of an old laboratory strain from 18 per cent. to 45 per cent. by passing it 41 times through rats and thus raising its killing powers in a standard dose from 20 per cent. to 86 per cent.

*Medium for growth and ways of sterilization.* It has been said that agar-grown cultures are less potent than broth-grown (Haffkine's method) (De Smidt, 1929; Naidu and Jung, 1929). Morison, Naidu and Avari (1924) had, however, got very similar protective effects when they used heat-killed broth and phenol- or heat-killed agar cultures in doses which

displayed equal degrees of toxicity for rats. Schütze's (1925) results with rats were of a similar order and when guinea-pigs were used he noted that the agar-grown cultures were superior to the broth-grown.

Sterilization is achieved in diverse ways, by heating, by antiseptic or by a combination of these. A recent innovation has been to bring about sterility by lysis with plague bacteriophage, followed by heating at 44° C. for 4 hours in the presence of 0·5 per cent. phenol (Flu, 1929). This method may possibly be less damaging to the antigen. It is not possible, however, to assess the relative merits of various types of vaccine until more experiments, planned on strictly comparative lines, have been performed. Rowland (1915) prepared from a serum-grown strain a nucleoprotein extract which protected rats against the more virulent serum-grown strain to as high a degree (75 per cent.) as did a broth-race extract when tested against a broth-race strain. Apparently, however, it is possible for the infectivity of a strain to outstrip the defensive powers of an extract prepared from it. Thus, Rowland's strain which in a dose of three million organisms normally killed 90 per cent., suddenly increased in virulence to such an extent that 100 per cent. of the controls succumbed and an extract prepared from it in this phase succeeded in saving only 22·5 per cent. of the rats. The extent to which an increase in test-dose can influence the survival rate among inoculated rats is afforded by Flu (1929). The vaccine employed by him was able to protect 91 per cent. of his rats against 40 m.l.d. but only 24 per cent. against 400 m.l.d.

In the preparation of vaccine consideration must be given to the fact that the immunogenic antigen in *B. pestis* is heat-labile. Also the toxicity of the product is of importance. In the case of the Haffkine prophylactic, e.g. it has been ascertained that storage at a temperature between 22 and 30° C. in the presence of 0·5 per cent. phenol brings about a diminution of its toxicity long before its protective power begins to decline. Naidu and Jung (1929) tested 10 different samples of Haffkine's prophylactic, inoculating 30 rats with each brew and giving each rat 0·5 c.cm., i.e.  $\frac{1}{6}$ th of a human dose. The test-dose was given 7 days after the immunizing dose and during this interval 14 of the 300 rats succumbed to the toxin. The remaining 286 rats were given a test-dose of living plague bacilli (0·003 mgm. of infected spleen pulp). Within the next 15 days 179 of the immunized animals succumbed to plague. Roughly about a third of the rats were protected. Better immunization has been reported by other workers (De Smidt, 1929; Flu, 1929) but it is quite likely that their results have been more apparent than real, there being no guarantee that the test-doses were as potent as the one used by Naidu and Jung in the above-cited experiment.

In Schütze's (1925) experiment with a serum-grown, phenol-killed vaccine, 91 per cent. of the inoculated rats survived, but, on the other hand, only 78 per cent. of the controls succumbed to the test-dose.

Though the results obtained so far in the laboratory may be disappointing, the statistics furnished by the use of plague vaccines in the field

## 94 PRODUCTION OF ACTIVE IMMUNITY TO BACTERIAL

indicate a considerable degree of efficacy. The latest figures given by Naidu and Jung (1929) for Haffkine's prophylactic in India may be roughly summarized as follows: Between 1897 and 1916 three million uninoculated gave an incidence rate of 1·8 per cent. and a case mortality of 85 per cent., while two million inoculated gave an incidence of 0·2 per cent. and a case mortality of 39 per cent.

### *Fowl cholera.*

It has been the usual experience that only the living attenuated organism is capable of conferring on fowls a solid immunity. Killed vaccines have proved ineffective. In pigeons, however, it is possible to secure a fairly solid immunity by killed antigen, and the contributions to this subject by Harvey and Iyengar (1928) are particularly worthy of mention as they throw light on many important points in prophylaxis by vaccines, including the degree and duration of the immunity. Their pigeons received two intravenous doses (0·04 mgm. and 0·08 mgm.) of heat-killed *P. aviseptica* at seven days interval, and the strain employed was one of very high virulence. For the relevant figures on various points the original must be consulted, but we may note here two sets of data, one on duration and degree of immunity and the other on the effect of re-immunization with fractions of an immunizing dose. These data show that the duration of immunity varies from 4 to 5 months, being shorter when the test-dose employed is greater. A falling immunity can be restored to normal by re-immunization with a small fraction of the usual immunizing dose and this newly conferred immunity lasts as long as the original. With Harvey and Iyengar's views as to the light thrown by these experiments with *Pasteurella* on prophylactic mechanisms generally we find ourselves in excellent agreement.

### *Virus Diseases.*

The results which have been achieved with killed virus, experimentally at least, though in some cases also in field trial, are detailed in the chapters dealing with rabies, foot-and-mouth disease, dog distemper, fowl-plague, yellow fever and herpes (Vol. VII). So intensive, indeed, have been the investigations in this sphere, particularly in two instances, experimental foot-and-mouth disease in guinea-pigs and distemper of dogs and ferrets, that the data collected illuminate the general problem of active immunity not only to viruses but to bacteria as well, and this position has been reached even though none of the viruses concerned have been artificially cultivated.

The immunization processes which have been employed to effect some measure of solid immunity in all the above-mentioned virus diseases have this one feature in common, viz. the use of a chemical agent (as a rule, phenol or formalin) which destroys the vitality of the virus without injuring materially its protective value as an antigen. The choice of chemical appears to be of some importance, though it is not yet clear

why in one case phenol is the more efficient and in another formalin. Further research may show a greater interchangeability of these two substances for this special purpose.

*Rabies.* The work of Fermi (1908) and of Semple (1911) led to the substitution in many countries of a phenolized rabies vaccine for the Pasteurian attenuated virus. Semple showed that 1 per cent. carbolic, acting at 37° C. for 24 hours on an 8 per cent. suspension of virus, yielded an inactive mixture when tested subdurally on rabbits, and the material he employed for immunization of rabbits, monkeys and dogs (small numbers only of each) was a 4 per cent. killed virus suspension in 0·5 per cent. phenol. The scheme of immunization practised in these animals involved daily and increasing doses of vaccine over a period of 3 or 4 weeks and the test-dose was given subdurally a fortnight after the last immunizing dose. A considerable degree of immunity resulted in all three species of animals. Stuart and Krikorian (1925) record that rabbits inoculated daily for 14 days with carbolized vaccine prepared from rabbit-brain resisted in 3 cases out of 5 a dose of 0·2 c.cm. of 1 per cent. fixed virus, while the remaining 2 succumbed after a prolonged period of incubation. They note also that one of the survivors in this experiment succumbed after the normal incubation period to a similar dose administered intradurally 14 months later. The duration of immunity conferred by the dead virus followed by the living fixed virus would appear, therefore, to be very limited. It may be noted that this form of massive immunization, which appears to be necessary to achieve immunity against subdurally inoculated living virus, has no parallel, except perhaps in those experimental efforts to produce immunity to poliomyelitis virus in monkeys. Recently it has been claimed by Rhoads (1930) that one large dose of this latter virus when distributed at one stage in many different portions of the dermis may confer on monkeys a solid immunity. This method, if its claims are confirmed, may be capable of wider application.

Formolized virus has also been used successfully in the immunization of dogs (Plantureux, 1926), so that in the case of rabies virus at least formol and phenol would both appear to be satisfactory reagents for inactivating the virus. Attention may also be drawn to the work of Cunningham and Malone (1930) on the comparative value as prophylactic agents, of carbolized and etherized rabies vaccines.

With regard to nervous complications and paralytic accidents in the course of antirabic treatment, experience has shown fairly clearly that with carbolized virus such accidents are very rare (see Stuart and Krikorian, 1928).

*Foot-and-mouth disease and distemper of dogs and ferrets.* Recent studies in active immunization against these viruses reveal many features of general interest. The work of the Foot-and-Mouth Disease Research Committee has shown that guinea-pigs inoculated with formolized virus prepared from vesicle fluid of guinea-pig's pads are in a proportion of

## 96 PRODUCTION OF ACTIVE IMMUNITY TO BACTERIAL

cases rendered immune to a subsequent dose of living virus in the sense that generalization of the virus throughout the body from the site of inoculation is in abeyance. The immunity is not sufficient to prevent the formation of a local vesicle when the test-dose is given in the pad. If the test-dose is given intramuscularly there is no lesion local or general.

Elsewhere (Vol. VII, p. 73) will be found details of preparation of the vaccine and of the numerous experiments designed to test the effect of altering the pH of the virus-containing fluid, the period of contact with formalin and the temperature at which contact is maintained. Slight changes in these particulars may have a profound effect on the protective value of the vaccine. The experiments also demonstrate clearly that the degree of immunity as tested by varying the amount of the living test-dose varies directly with the dose of the immunizing antigen. So far as these experiments have gone, the duration of the immunity would not appear to exceed 3 to 6 months, but as in Harvey and Iyengar's experiments with *Pasteurella* (cited above), it was found that minute doses of foot-and-mouth antigen were capable of restoring a falling immunity to its pristine level. From the work of Laidlaw and Dunkin (1928) on distemper of dogs and ferrets we learn also that the value of a formolized distemper vaccine depends largely on the concentration of virus in the original fluid. Vaccine prepared from dog-distemper tissues was found to confer a low-grade protection on ferrets, whereas that made from ferret material was only moderately successful for dogs. This curious result the authors would attribute to interference with development of protective antibodies by other antibodies arising in response to the heterologous protein.

Though a very definite resistance to the living virus was conferred on dogs by the inactivated product, it was considered advisable for field purposes to convert this temporary form of protection to one of solid immunity by giving a final dose of the living virus.

### ORAL METHODS OF IMMUNIZATION.

In preceding sections we have been concerned only with the production of immunity by vaccines administered for the most part subcutaneously. It is necessary, however, to take cognizance of practices which involve the oral administration of killed vaccine, though at present only tentative efforts in this direction are on record. Naturally such practices, if proved to be efficient, would remove many of the difficulties and prejudices met with in the course of mass inoculations in the field by the syringe.

It has long been known that the introduction of antigen by the mouth may be followed by low-grade antibody development due either to failure of antigen to be absorbed into the general circulation or to destruction of a major part of it before it reaches an absorbing surface.

Elsewhere (Local Immunity, Chapter II) are discussed Besredka's claims by means of orally administered antigen so to sensitize the intestinal

mucosa that it no longer functions as the one and only tissue susceptible to attack by organisms such as *B. typhosus* and *B. dysenteriae*. That an enterotropism of *B. typhosus* exists in the sense postulated by Besredka has received little or no support from subsequent experimental work. Reference may be made, for example, to the detailed experiments by Ørskov and Moltke (1928) on the fate of *Salmonella* organisms introduced into mice *per os*. These authors find the organisms first in the regional lymph glands of the intestine, whence by the blood they reach the liver and spleen, to appear at a later stage in the intestine. We are concerned here, however, mainly with evidence indicating the value of orally administered antigen for prophylactic purposes and less with the theoretical basis of the method. The relevant data on this point are concerned almost solely with enteric disease, bacillary dysentery and cholera, and only those dealing with experimental work deserve notice at the present time. It is true that certain field data in connection with orally administered typhoid antigen are on record, but they do not make an impressive picture from a statistical standpoint. For full summaries of experimental work in this sphere the reader may be referred to papers by Enlows (1925) and Otten and Kirschner (1927) which deal chiefly with bacillary dysentery. These papers, together with those of Thomson (1916), Kanai (1921), Nicolle and Conseil (1922), Fränkel (1928) and Blanc and Caminopetros (1928), furnish data which demonstrate clearly the production of solid immunity in rabbits, mice, and men, by variously prepared and variously administered vaccines of *B. dysenteriae* (Shiga). A survey of these records shows (1) that rabbits can be immunized by killed 'Shiga' administered orally so as to withstand infection by the living organism inoculated intravenously; (2) that the degree of immunity conferred is on the whole of a somewhat lower order than that conferred by the subcutaneously administered vaccine (Kanai, Enlows); (3) that the oral method has some advantage in rabbits in that casualties during immunization are less frequent (Enlows); (4) that clear evidence of antibody production following oral immunization is obtainable (Kanai, Otten and Kirschner)—a fact which would suggest that there is no inherent difference in the type of immunity produced by the two procedures; (5) that while oral administration by Nicolle and Conseil of killed 'Shiga' to human volunteers protected the latter from contracting dysentery following oral administration of the living *B. dysenteriae* (Shiga), the results of Blanc and Caminopetros were not favourable to oral immunization. The latter authors, indeed, obtained the best protection in human volunteers by immunization with the living 'Shiga' subcutaneously administered, though the killed 'Shiga' by the same route also conferred a considerable degree of protection. The vaccines used by Blanc and Caminopetros were stated to be non-toxic. The organism was grown on dry media for 24 hours, when the growth was removed and employed as vaccine 'before autolysis took place'. Provided the toxicity of the vaccine can be annulled without affecting the protective value of the

## 98 PRODUCTION OF ACTIVE IMMUNITY TO BACTERIAL

vaccine there is no doubt that the subcutaneous method for human use would be the method of election, and it is possible that further experiments with formolized vaccines on lines suggested by Otten and Kirschner may lead to the development of a satisfactory antigen for prophylaxis against bacillary dysentery.

Until the problem of toxicity is satisfactorily solved it would seem reasonable to afford the fullest trial to the oral method even with the disadvantages that attend it in the matter of uncertainty of dosage and diminished chance of yielding the higher grades of immunity.

### Active Immunization in the Light of Recently Acquired Knowledge of Bacterial Antigen.

With the recognition of the complexity of antigens that exists within the bacterial body has come a more complete understanding of immunity. Smith and Reagh (1903), Weil and Felix (1917), Arkwright (1920), Avery and Heidelberger (1925), together with other workers, have demonstrated the presence of flagellar and somatic, of specific carbohydrate and non-specific protein constituents in a variety of organisms. Although the part which these components of the bacterial cell play in the processes of immunity is still the subject of investigation, one's views have already been considerably modified by the knowledge gained.

To take first the case of the typhoid-salmonella group of bacteria, it has been proved for many of them that when motile, as they for the most part are, they possess a labile antigen which is associated with their flagella, and, by analogy with the motile *Hauch*-producing *B. proteus* forms of Weil and Felix, has been called the H antigen ; when the bacilli are non-motile, this H antigen is absent. Whether motile or not, the body of the bacterium contains antigen which is more stable towards heat and other influences than the H antigen and has been called, by similar analogy with the *ohne-Hauch* forms of *B. proteus*, the O antigen.

Both of these antigens may be present in one of two states ; Andrewes (1922) has shown that the H antigen of many bacteria may occur in either a non-specific or specific phase, the distinction residing in the serological relationship it bears or does not bear to other nearly related types ; Arkwright (1920) has demonstrated that the somatic antigen may exist either in the normal or what he called from colonial appearances, the smooth form, or in an abnormal form, called, for similar reasons, rough ; these two antigens have, therefore, been labelled S and R respectively, and according to White (1928, 1929) R represents the protein fraction of the bacterial body deprived of its acid-soluble factor, while S represents the somatic antigen when still in possession of that factor.

With this knowledge of the constitution of the cell and its possible variation, experiments have been devised to determine which factors are concerned in the creation of active immunity. Arkwright (1927), in a series of experiments upon guinea-pigs, concluded that in the case of

immunity to both *B. paratyphosus A* and *B. typhosus* the effective portion of a prophylactic vaccine was the S or smooth somatic antigen which it contained ; the use of a motile form with consequent incorporation of H antigen did not provide a vaccine with better protective powers than did the use of a non-motile form ; the heating of an S vaccine to 100° C., which would destroy the antigenic efficacy of H, were traces of it present, did not lower the prophylactic value of that vaccine ; but if an R form is substituted for an S form, the value of a vaccine prepared therefrom sinks very notably.

The importance of S somatic antigen is also plainly indicated in the experimental results of Ibrahim and Schütze (1928) and of Topley (1929), both of which were carried out on mice, the organism used being *B. aertrycke*. Ibrahim and Schütze demonstrated the survival of 50 per cent. of their animals after the administration of an H + S vaccine and 7 per cent. when the vaccine had contained no S but merely H + R. Topley worked with large groups of 200 to 400 mice, kept in a common run and subjected to the risks of natural infection from an epizootic initiated by the addition of infected animals ; he estimated the daily specific death-rate of the various groups and showed that inoculation with an H + S vaccine lowered the average daily death-rate to 66 per cent. of the death-rate of the control mice, while inoculation with an H + R vaccine only lowered the death-rate to 83 per cent., a degree of protection that might well be non-specific, for vaccines prepared from a rough typhoid strain and a *Staphylococcus*, both organisms without any antigenic relationship to *B. aertrycke*, did very much the same, their average daily death-rate being 85 per cent. of that of the controls.

The prophylactic value of somatic antigen is definitely established by these experiments. Without the presence of that antigen vaccines produce no specific immunity. It is not even necessary that the antigen shall be derived from the same bacterial species as that against which protection is desired. *B. paratyphosus B* and *B. aertrycke*, for example, share the same somatic antigen. Topley, in the above-cited experiments, used *B. paratyphosus B* in the vaccination of one group, the infecting organism being, as mentioned, *B. aertrycke* ; the average daily death-rate for this group was 65 per cent. of the death-rate of the controls, a figure practically identical with that of the aertrycke-immunized mice. Schütze (1930) repeated the experiment, using the intraperitoneal route for inoculation of the infective test-dose, and obtained a similar result. Herzog and Schiff (1922), tracing the course of a food-poisoning outbreak due to infection with *B. enteritidis* Gaertner which occurred in a hospital containing typhoid convalescents, noticed that these latter escaped the illness, the only patients partaking of the food to do so. As *B. typhosus* and *B. enteritidis* Gaertner have a common S antigen, it is reasonable to suppose that the immunity observed was dependent on that factor. In an experiment on mice, Schütze (1930) was able to confirm this observation.

The resistance of inoculated animals to infection has by many been ascribed to the antibodies which the inoculum occasions. For this reason, the prophylactic importance of somatic antigen being recognized, efforts have been made to assess the degree of immunity by estimation of the S antibody titre of the circulating blood. This titre is most conveniently measured in terms of agglutinin units, and it is, therefore, by means of the agglutination test that attempts to correlate protection with antibody have been carried out.

Topley demonstrated the presence of S agglutinins in all four of his groups inoculated with vaccines containing S antigen, but only a very small proportion of the animals possessed titres even as low as 1 in 20; 10 out of the 52 tested in one group was the biggest return, 1 out of 66 in another group the lowest. Such differences in the agglutination response of the groups did not correspond with similar differences in immunity, for all had fairly equal degrees of resistance to infection.

Schütze, who gave his mice one more inoculation than Topley, and larger doses, found an S titre of 1 in 100 and higher in 45 out of the 111 mice tested, 16 mice reaching 1 in 200 and higher, while 5 mice achieved 1 in 400. There was no correlation between the average titre of an S-inoculated group and its resistance to infection. A group which for some reason had not reacted by producing much agglutinin, might still display an immunity as pronounced as that of a group with a good agglutinin response.

It is clear from both of these experiments that the antibody response of a group of mice is no criterion of its resistance to infection; it is possible that in those animals which evince immunity without notable titres of antibody in their blood, antibodies may be present in minimal quantities or they may be sessile, preformed and ready for release at the onset of infection, or the tissues, sensitized by inoculation, may possess a potential antibody-producing capacity which exceeds that of normal uninoculated animals and is demonstrated as soon as infection occurs; there may even be a state of immunity quite unconnected with antibody response.

Felix (1924) and Gardner (1929) have pointed out with what difficulty somatic antibodies are formed by man after inoculation with typhoid and paratyphoid vaccines; in this respect man seems to resemble the mouse, while the rabbit, on the other hand, responds much better, though the difference in the size of the inoculum usually given to the two species, in respect of their size, must certainly be taken into account.

Although man produces somatic antibodies with difficulty there is no reason to suppose that a bad response in this respect indicates a lack of immunity any more than it does in the mouse; all that one can say is that it is impossible to assess the degree of immunity by the in-vitro test.

But this does not necessarily mean that those animals which possess circulating antibodies may not be on the average in a better state of protection than those which do not; indeed, from the figures which are

available, it is very likely that they are. The following table gives results published by Schütze (1930) :

*S Titre versus Death-Rate for Mice immunized with B. aertrycke.*

Days after Test Dose.	Deaths among Mice with Titres less than 1/100.	Deaths among Mice with Titres 1/100 to 1/400.
4	6 out of 66 = 9 per cent.	0 out of 45 = 0 per cent.
14	17 .. 66 = 26 ..	7 .. 45 = 16 ..
32	25 .. 66 = 38 ..	14 .. 45 = 31 ..

Average length of life of 25 mice with titres less than 1/100 = 11.4 days.

Average length of life of 14 mice with titres 1/100 to 1/400 = 15.1 days.

It would appear that over and above a basal immunity not detectable by in-vitro tests there is an extension of it, probably dependent on circulating antibody, to which the S agglutinin titre may serve as a rough index.

So far we have been concerned with immunity to organisms of the typhoid-salmonella group.

Since Dochez and Avery (1917) discovered the existence of a soluble specific substance in pneumococci, American workers have analysed the antigenic complex of this group of organisms and investigated the bearing of the several constituents upon the immunity that can be conferred by inoculation. It has been possible to differentiate two substances in the pneumococcus cell; a polysaccharide which is associated with the capsule and a protein constituent representing the body of the cell (Heidelberger and Avery, 1923). According to Gaspari and his co-workers (1928) the protein fraction is less stable than the polysaccharide; heating to 55° C. for 30 minutes caused a marked decrease in the antibody-invoking effectiveness of the former. They agreed with Dochez and Avery (1917) and others who found the polysaccharide resistant to boiling and to the action of enzymes and other chemicals. Antibodies (agglutinins and precipitins) can be prepared for both of these (protein and carbohydrate) by animal inoculation, and serological tests demonstrate that while the protein substance is merely species-specific, being common to all types, the polysaccharide is type-specific, serological differences between the types being confirmed by the discovery of corresponding chemical differences in the purified carbohydrate extracts. In agreement with these findings two kinds of immunity have been postulated, one related to the protein fraction and one related to the soluble specific substance.

Inoculation with degraded avirulent forms (Tillett, 1928) which lack the carbohydrate-containing capsule or with the chemically separated protein fraction of normal encapsulated forms (Perlzweig and Keefer, 1925) will confer immunity against all pneumococcal types by virtue of the community of protein substance possessed by the whole species.

An immunity limited to one pneumococcal type is obtainable by inoculation with vaccine prepared from a capsulated strain of that type ; it is then a completely specific protection, confined to the pneumococcal type from which the vaccine has been prepared. To achieve this it has been held necessary to use the intact cell and not solutions thereof or the purified carbohydrate itself. In this latter form the polysaccharide fraction is non-antigenic ; only when it is intimately associated with the protein fraction does it become antigenic and capable of evoking the formation of antibodies and immunity for itself. Schiemann and Casper (1927), however, assert that they succeeded in the production of type-specific immunity in mice by the injection of protein-free carbohydrate derived from Types I and III, and Barach (1928) reported a similar result, using a water extract of pneumococcus, Types I and II, freed of cellular elements by filtration. Unfortunately in neither case were in-vitro experiments performed to demonstrate the presence or absence of antibodies, though Barach inferred the presence of carbohydrate antibodies and the absence of protein antibodies from the fact that the sera of rabbits inoculated with his filtrates conferred a type-specific passive immunity upon mice but no cross immunity between the types, while Schiemann (1929) demonstrated the production of specific protective bodies in the serum of mice inoculated with carbohydrate but not in the serum of similarly inoculated rabbits.

The virtue of antibodies in the processes of immunity has been much disputed and cannot be regarded as settled. Armstrong (1925) compared the antibody response in the rabbit and the rise in resistance to infection in the mouse, and, finding the course of these to correspond almost exactly, considered the amount of antibody to be 'a faithful index of the degree of resistance to infection of the animal as a whole'. Cecil and Steffen (1923) succeeded in protecting monkeys with Type II, III and IV vaccines without the production of antibodies protective to mice. This lack of antibodies may have been illusory, for if the immunity conferred was an antiprotein one the serum would contain protein antibodies, which, as Tillett (1928) has shown, are incapable of conferring passive immunity upon mice, though in this case there would have to be active cross immunity among the monkeys, and this is uncertain as the point was not tested. On a previous occasion Cecil and Blake (1920) had succeeded in demonstrating some cross immunity between the types, which no doubt was antiprotein in origin.

Wright (1927), working with Type I pneumococci, came to the conclusion that the chief effect of immunization was an increase in the opsonic powers of the body fluids, but he did not feel justified in excluding some accompanying cellular immunity. Months after the demonstration of antibodies in the serum of an immunized animal had ceased to be possible, the whole blood of such an animal was found to be inimical to the in-vitro growth of the corresponding pneumococcal type ; this might well be due to the presence of undetectable traces of antibody still present in

the blood. He specially instanced one rabbit in which, eleven months after immunization, this inhibitory power *in vitro* was absent and yet *in vivo* the capacity of destroying pneumococci still existed ; it is impossible to say whether this residual immunity is due to antibodies still present in the tissues, though absent, for practical purposes, from the blood, or is the result of an increased activity of the tissues alone, a purely cellular immunity. Wright was not able to interfere with the power of an immunized animal to remove pneumococci from its blood by blockading the reticulo-endothelial system, but Singer and Adler (1924) claim to have done so for Types I, II and III and regard these tissues as playing an important part, indeed in the case of Type III the only part, in pneumococcal immunity.

One must conclude that in the case of the pneumococcus, as we have already seen in that of the salmonella-typhoid group, protection by means of vaccines depends not only on the presence of circulating antibodies but also upon further factors whose nature, whether humoral or cellular, it is at present impossible to define.

### Concluding Remarks.

As will have been gathered from the scope of the preceding sections, active immunization has found its principal field of usefulness in the control of those epidemic diseases of man and animals in which sanitary science finds itself incapable of attacking effectively the infected sources or of controlling the spread of the infective agent. The assessment of its value in terms of incidence or mortality among the vaccinated population has perhaps in few cases only been capable of expression in terms likely to satisfy statisticians who must take cognizance of many factors, whether of environment or of natural resistance, which may influence such assessment.

It may be taken as established that individuals differ in their capacity to respond to active immunization. Also the degree and distribution in a particular population of natural resistance to an infective agent are not readily expressed numerically. It is probable, therefore, that the statistical justification for active immunization procedures, whether in man or animals, may have in the future to rest mainly on laboratory data concerning animal populations in which the range of normal resistance, the hereditary antecedents, the dietetic factors, &c., are fairly precisely known.

The accumulation of data of this character may help to clarify the problem of herd immunity in the minds of those at least who do not accept the principle that what is worth trying for the individual is worth trying for all.

### REFERENCES.

- ANDREWES, F. W., 1922, *J. Path. Bact.*, **25**, 505.
- ARKWRIGHT, J. A., 1920, *J. Path. Bact.*, **23**, 358 ; 1927, *ibid.*, **30**, 345.
- ARMSTRONG, R. R., 1925, *Proc. R. Soc., B*, **98**, 525.
- AVERY, O. T. & HEIDELBERGER, M., 1925, *J. Exp. Med.*, **42**, 367.
- BARACH, A. L., 1928, *J. Exp. Med.*, **48**, 83.
- BESREDKA, A., 1919, *Ann. Inst. Pasteur*, **33**, 301, 882.

# 104 PRODUCTION OF ACTIVE IMMUNITY TO BACTERIAL

BLANC, G. & CAMINOPETROS, J., 1927, *C.R. Acad. Sci.*, Paris, **185**, 1625; 1928, *Rev. Hyg. Méd. Prév.*, **50**, 321.

BROUGHTON-ALCOCK, W., 1912, *Lancet*, Lond., ii, 504.

CASTELLANI, A., 1913, *Lancet*, Lond., i, 595.

CECIL, R. L. & BLAKE, F. G., 1920, *J. Exp. Med.*, **31**, 657, 685.

CECIL, R. L. & STEFFEN, G. I., 1923, *J. Exp. Med.*, **38**, 149.

COLE, R. L., 1904, *Z. Hyg. InfektKr.*, **46**, 371.

CUNNINGHAM, J. & MALONE, R. H., 1930, *Ind. Med. Res. Mem.*, No. 15, Thacker, Spink & Co., Calcutta.

DE SMIDT, F. P. G., 1929, *J. Hyg.*, Camb., **29**, 201.

DOCHEZ, A. R. & AVERY, O. T., 1917, *J. Exp. Med.*, **26**, 477.

ENLOWS, E. M. A., 1925, *Publ. Hlth. Rep.*, Wash., **40**, 639.

ENLOWS, E. M. A. & BROOKS, S. C., 1928, *J. Lab. Clin. Med.*, **14**, 103.

FELIX, A., 1924, *J. Immunol.*, **9**, 115.

FERMI, C., 1908, *Z. Hyg. InfektKr.*, **58**, 233.

FLU, P. C., 1929, *Zbl. Bakt.*, Abt. I, Orig., **113**, 473.

FRÄNKEL, E., 1928, *Z. ImmunForsch.*, **59**, 416.

FRIEDBERGER, E., 1919, *Z. ImmunForsch.*, Tl. I, Orig., **28**, 119; 1927, *Zbl. Bakt.*, Abt. I, Orig., **104**, Beilage 181.\*

GARDNER, A. D., 1929, *J. Hyg.*, Camb., **28**, 376.

GASPARI, E. L., SUGG, J. Y., FLEMING, W. L. & NEILL, J. M., 1928, *J. Exp. Med.*, **47**, 131.

GREENWOOD, M. & YULE, G. U., 1915, *Proc. R. Soc. Med.*, **8** (Sect. Epidem.), 113.

HAFFKINE, W. M., 1892, *C.R. Soc. Biol.*, Paris, 9 s., **4**, 740; 1913, *Protective Inoculation against Cholera*, Thacker, Spink & Co., Calcutta.

HARVEY, W. F. & IYENGAR, K. R. K., 1923, *Ind. J. Med. Res.*, **10**, 990, 1147; **11**, 113, 433; 1928, *ibid.*, **15**, 935.

HEIDELBERGER, M. & AVERY, O. T., 1923, *J. Exp. Med.*, **38**, 73.

HERZOG, F. & SCHIFF, F., 1922, *Z. ges. exp. Med.*, **29**, 412.

HETSCH, 1904, *Handb. d. path. Mikroorg.*, hrsg. Kolle u. Wassermann, Jena, **4**, 1094.

HUTYRA, F. & MAREK, J., 1910, *Spezielle Pathologie u. Therapie der Haustiere*, 3rd ed., G. Fischer, Jena.

IBRAHIM, H. M. & SCHÜTZE, H., 1928, *Brit. J. Exp. Path.*, **9**, 353.

JENNER, E., 1798, Citations from 1889, *History and Pathology of Vaccination*, E. M. Crookshank, H. K. Lewis, London.

KANAI, S., 1921, *Brit. J. Exp. Path.*, **2**, 256.

KOLLE, W. & OTTO, R., 1903, *Z. Hyg. InfektKr.*, **45**, 507.

LAIDLAW, P. P. & DUNKIN, G. W., 1928, *J. Comp. Path.*, **41**, 209.

MEINICKE, E., JAFFÉ, J. & FLEMMING, J., 1906, *Z. Hyg. InfektKr.*, **52**, 416.

METCHNIKOFF, E. & BESREDKA, A., 1911, *Ann. Inst. Pasteur*, **25**, 193; 1912, *C.R. Acad. Sci.*, Paris, **155**, 112.

MIYASHITA, K., 1929, *Z. ImmunForsch.*, **64**, 138.

MORISON, J., NAIDU, B. & AVARI, C. R., 1924, *Ind. J. Med. Res.*, **12**, 313, 321.

MURATA, N., 1904, *Zbl. Bakt.*, Abt. I, Orig., **35**, 605.

NAIDU, B. P. B. & JUNG, J. S., 1929, *Ind. J. Med. Res.*, **17**, 199.

NAIDU, B. P. B., MALONE, R. H. & AVARI, C. R., 1926, *Ind. J. Med. Res.*, **18**, 823.

NICOLLE, C. & CONSEIL, E., 1922, *Ann. Inst. Pasteur*, **36**, 579.

NICOLLE, C., CONOR, A. & CONSEIL, E., 1912, *C.R. Acad. Sci.*, Paris, **154**, 1823.

NIJLAND, A. H., 1911, 1913, cited by Greenwood and Yule (loc. cit.).

ØRSKOV, J. & MOLTKE, O., 1928, *Z. ImmunForsch.*, **59**, 357.

OTTEM, L. & KIRSCHNER, L., 1927, *Z. Hyg. InfektKr.*, **107**, 314.

PASTEUR, L., 1880<sup>1</sup>, *Bull. Acad. Méd.*, Paris, 2 s., **9**, 121, 390, 1119; 1880<sup>2</sup>, *ibid.*, 2 s., **9**, 682; 1882, *C.R. Acad. Sci.*, Paris, **95**, 1250; 1883, *ibid.*, **96**, 979; 1885, *ibid.*, **101**, 765; 1886, *ibid.*, **103**, 777.

PASTEUR, L. et al., 1880, *Bull. Acad. Méd.*, Paris, 2 s., **9**, 983; 1881<sup>1</sup>, *C.R. Acad. Sci.*, Paris, **92**, 429, 666, 1378; 1881<sup>2</sup>, *ibid.*, **92**, 1259; 1882<sup>1</sup>, *ibid.*, **95**, 1187; 1882<sup>2</sup>, *ibid.*, **95**, 1120; 1884, *ibid.*, **98**, 457, 1229.

PASTEUR & THUILIER, 1883, *C.R. Acad. Sci.*, Paris, **97**, 1163.

PERLZWEIG, W. A. & KEEFER, C. S., 1925, *J. Exp. Med.*, **42**, 747.

PFEIFFER, R. & FRIEDBERGER, E., 1902, *Berl. klin. Wschr.*, **39**, 581.

PFEIFFER, R. & KOLLE, W., 1896, *Z. Hyg. InfektKr.*, **21**, 203.

PLANTUREUX, E., 1926, *C.R. Acad. Sci.*, Paris, **182**, 1578; *Rev. gén. Méd. vét.*, **35**, 619.

POWELL, A., 1896, *Lancet*, Lond., ii, 171.

REPORT OF THE ANTITYPHOID COMMITTEE, 1912.

RHOADS, C. P., 1930, *J. Exp. Med.*, **51**, 1.

ROWLAND, S., 1915, *J. Hyg., Camb.*, Plague Supplement IV, 752, 759, 761.

RUSSELL, A. J. H., 1927, *Trans. 7th Cong. Far East. Assoc. Trop. Med.*, **1**, p. 523.

SAVAS, C., 1914, *Wien. klin. Wschr.*, **27**, 1093.

SCHIEMANN, O., 1929, *Z. Hyg. InfektKr.*, **110**, 567.

SCHIEMANN, O. & KASPER, W., 1927, *Z. Hyg. InfektKr.*, **108**, 220.

SCHNÜRER, 1927, *Zbl. Bakt.*, Abt. I, Orig., **104**, Beiheft 115.\*

SCHÜTZE, H., 1925, *Brit. J. Exp. Path.*, **6**, 207; 1930, *ibid.*, **11**, 34.

SEMPLE, D., 1911, *Sci. Mem. Med. and San. Dept. Govt. India*, New Ser., No. 43.

SINGER, E. & ADLER, H., 1924, *Z. ImmunForsch.*, **41**, 71, 468.

SMITH, T. & REAGH, A. L., 1903, *J. Med. Res.*, **10**, 89.

STRONG, R. P., 1904, *Pub. Bur. Sci. (Gov. Labs.) Manila*; (No. 16), 1906, *Philipp. J. Sci.*, **1**, 181; 1907, *ibid.*, **2**, 155.

STUART, G. & KRIKORIAN, K. S., 1925, *Ann. Trop. Med. Parasitol.*, **19**, 391; 1928, *ibid.*, **22**, 327.

THOMSON, J. D., 1916, *Brit. Med. J.*, i, 303.

TILLETT, W. S., 1928, *J. Exp. Med.*, **48**, 791.

TOPLEY, W. W. C., 1929, *Lancet*, Lond., i, 1337.

TOUSSAINT, 1880, *Bull. Acad. Méd.*, Paris, 2 s., **9**, 942.

WASSERMANN, 1903, *Festschrift Robert Koch*, 503.

WEBER, R., 1916, *Z. Hyg. InfektKr.*, **82**, 351; 1917, *ibid.*, **84**, 425.

WEIGMANN, F., 1926, *Z. Hyg. InfektKr.*, **108**, 650.

WEIL, E. & FELIX, A., 1917, *Wien. klin. Wschr.*, **30**, 1509.

WHITE, P. B., 1928, *J. Path. Bact.*, **31**, 423; 1929, *ibid.*, **32**, 85.

WILLEMS, 1852, cited by Hutyra and Marek (loc. cit., Bd. I, 399).

WRIGHT, H. D., 1927, *J. Path. Bact.*, **30**, 185.

## CHAPTER IV. ACTIVE IMMUNIZATION WITH TOXIN.

BY A. T. GLENNY (WELLCOME PHYSIOLOGICAL RESEARCH LABORATORIES).

### Methods of Testing Toxin and Units of Measurement.

THERE are two constituents of filtered broth cultures of *C. diphtheriae* that can be measured by in-vivo and in-vitro tests, namely, toxin and toxoid. The word toxoid is here used in a general sense for any modification of specific toxin which produces no symptoms in guinea-pigs but is still capable of combining with antitoxin. The amount of specific toxin present in any preparation may be measured by its action upon guinea-pigs (see Toxicity Tests, p. 108). There is, however, no direct method of measuring toxoid content alone, but the total content of toxin plus toxoid, i.e. the total amount of specific antigen, is measured by the amount of antitoxin with which it will combine (see Combining Power Tests, p. 111). Table I is a summary of the different methods of testing used in the titration of toxin and toxoid.

TABLE I.  
Units of Measurement of Diphtheria Toxin.

Method.	Measures of Specific Toxicity, i.e. Toxin Content.	Measures of combining Power with 1 Unit of Antitoxin, i.e. Toxin + Toxoid Content.	Amounts of a Typical Toxin (c.cm.).	Reaction.
<i>In vivo.</i> Subcutaneous ..	m.l.d.		0.003	Kills guinea-pigs in 5 days.
Intracutaneous ..	m.r.d.		0.000002	Causes minimal skin reactions.
Subcutaneous ..		Lo	0.180	No local oedema.
Subcutaneous ..		L+	0.210	Kills in 5 days.
Intracutaneous ..		Lr	0.175	Causes minimal skin reactions.
<i>In vitro.</i> Flocculation ..		Lf	0.155	Flocculates most rapidly.

#### DEFINITIONS OF UNITS.

*The minimal lethal dose (m.l.d.)* of toxin is the least quantity of toxin which, given subcutaneously, will kill within five days the majority of guinea-pigs (250 gm. body-weight) injected.

*The minimal reacting dose (m.r.d.)* of toxin is the least quantity of toxin which, injected intracutaneously into a guinea-pig, will cause a reaction in the form of a small red flush 5 mm. in diameter visible 36 hours after injection.

*The unit of antitoxin* is that amount which is contained in certain known weights of dried standard antitoxin preserved at the Institut für experimentelle Therapie, Frankfurt, Germany; the Hygiene Laboratory, U.S. Public Health Service, Washington; and the National Institute for Medical Research, London. The unit was originally chosen as that amount of antitoxin which completely neutralized 100 m.l.d. of a certain toxin. This unit remains the fundamental basis of comparison of antitoxic values of sera and combining powers of toxin.

*The Lo (nul limit)* dose of toxin is the greatest amount of toxin which causes no local oedema when mixed with one unit of antitoxin and injected subcutaneously into a guinea-pig.

*The L+ (limit of death)* dose of toxin is the least amount of toxin which, when mixed with one unit of antitoxin and injected subcutaneously into 250 gm. guinea-pigs, kills within five days.

*The Lr (limit of reaction)* dose of toxin is the least amount of toxin which, when mixed with one unit of antitoxin, forms a mixture of which 0.2 c.cm. injected intracutaneously into guinea-pigs causes a minimal skin reaction.

*The Lf (limit of flocculation)* dose of toxin is the amount of toxin per unit of a certain chosen typical antitoxin in the mixture that flocculates more rapidly under the same conditions than all other mixtures containing other amounts of the same toxin per unit of the same antitoxin.

#### *Subsidiary units.*

*The in-vitro equivalent of antitoxin* is the equivalent of one Lf dose of toxin tested by the flocculation method. By definition, the in-vivo unit and the in-vitro equivalent are the same for certain sera but the subsidiary in-vitro equivalent is necessary because atypical sera are found of which the in-vitro and in-vivo titrations do not agree (cf. p. 121).

*The Lr/500 dose of toxin* is the least amount of toxin which, when mixed with 1/500 unit of antitoxin and injected intracutaneously into a guinea-pig, causes a minimal skin reaction. Other doses, e.g. Lr/100, may be used for convenience. These smaller test-doses are chosen to prevent severe reaction or death of the test animal injected with under-neutralized mixtures.

*The Schick dose of toxin* was originally fixed as 1/50 m.l.d. of a mature toxin. The Schick dose is a test-dose to determine a certain level of antitoxin content in the blood or tissues of man. The following definition

is, therefore, suggested. The Schick dose of toxin is approximately 1/50th m.l.d. of a mature toxin ; the intracutaneous injection of 1/25th of a test-dose into a guinea-pig should always produce a well-marked reaction, and that of 1/40th should fail to produce a reaction ; 1/1,000th unit of antitoxin should just neutralize the test-dose.

#### TESTS FOR TOXICITY.

Determination of the minimal lethal dose of a toxin is of little practical value as a measure of its utility. The use of the m.l.d. has been particularly misleading in the past where it has been applied by many workers to the estimation of the strength of toxin in culture filtrates when the methods and conditions of preparing them are determined for the immunization of horses. The utility of a toxin depends upon the total amount of specific antigen (toxin + toxoid), and this can only be measured in terms of the amount of antitoxin with which it will combine. Misleading results have also been obtained by those workers who have measured the action of physical or chemical agents upon culture filtrates by determining the m.l.d.

*Seasonal variation.* Südmersen and Glenny (1910) have shown that the m.l.d. of a toxin varies with the time of year the tests are made. Less toxin is needed to kill a guinea-pig in winter than in summer. MacConkey (1912) found that the average strength of weekly batches of diphtheria toxin prepared by him showed similar variation.

*Individual variation.* Considerable variation is found in the time of death of animals injected with the same dose of toxin unless they are bred and kept under carefully standardized conditions. Trevan (1927) has calculated that if guinea-pigs of the same weight are taken at random about 50 must be used in each group when the toxicity of two samples of diphtheria toxin are compared for an error of  $\pm$  10 per cent. For a 20 per cent. error 11 to 12 animals in each group would suffice.

*Relation between fatal dose and weight of guinea-pig.* Dreyer and Walker (1914) claimed that the dose necessary to produce the same effect upon animals of different weights varied according to the surface areas of the animals and that these areas are in proportion to  $W^{0.72}$ . French workers assume that within reasonable limits, dose is directly proportional to weight ; thus Abt and Loiseau (1922), quoting the m.l.d. for a number of toxins determined upon guinea-pigs weighing 350 gm., stated that the number of m.l.d. per c.cm. so obtained should be multiplied by 1.4 in order to compare with similar figures obtained with guinea-pigs of 250 gm. Most experimental work, however, suggests that dose should be proportional to the two-thirds root of the body-weight, but no detailed work on the large number of guinea-pigs necessary for an exact comparison has yet been made.

*Relation between dose and death time.* Several formulæ have been suggested at different times, notably that of Dreyer and Walker (1914) :

$$\frac{1}{Do - a} - \frac{1}{Dl - a} = k (To - Tl),$$

which has been translated thus : 'To every equal increment in time there corresponds a definite decrease in

active dose'. This formula agrees fairly well with experimental data over a relatively narrow range, but it is not applicable when the extreme doses vary between 1 and 20 or more fatal doses. Better agreement over a wider scale is shown by the formula suggested by Glenny (1925<sup>1</sup>):  $(\log D - \log A)(T - b) = C$ , where D is the dose and A the theoretical m.l.d., i.e. the dose killing in infinite time, T is time of death in hours, b is the minimal lethal time, and C is a constant.

Reasonable accuracy of fit was obtained with a toxin with a m.l.d. of 0.002 c.cm. over a range of doses from 0.002 to 5 c.cm., and with a concentrated toxin with a m.l.d. of 0.00008 c.cm. over a range from this dose to 5 c.cm. With these two toxins the minimal lethal time was 10 hours and the value of the constant 12. With a weaker toxin (m.l.d., 0.2 c.cm.) the value of the constant was increased to 22, probably because increased non-specific effects delayed the rates of absorption.

*Lethal dose for different animals.* Animals must be considered in three groups: (1) those like guinea-pigs that have no natural immunity and are particularly susceptible to diphtheria toxin, (2) those like horses and men that may have natural immunity, (3) those like mice and rats that, without possessing immunity, have a natural resistance to diphtheria toxin not due to circulating antitoxin.

Guinea-pigs, rabbits and pigeons appear to have a somewhat similar degree of susceptibility to diphtheria toxin if allowance be made for weight. It is the absence of natural immunity that makes these animals of such particular use for testing diphtheria toxin. Schmidt (1928<sup>1</sup>) found that the subcutaneous m.l.d. for a guinea-pig was the same as the intravenous m.l.d. for a rabbit weighing 2,000 gm. Koulikoff and Smirnoff (1927) used pigeons for testing the m.l.d. of toxins. Koulikoff (personal communication) states that pigeons have been extensively used in Russia for many years for this purpose. Ramon, Noureddine and Erber (1928) give the m.l.d. for a pigeon of 250 gm. as twice that of a guinea-pig of the same weight. In the author's experience, the m.l.d. for these two animals is approximately the same.

The lethal dose for many animals varies with their degree of natural immunity. A horse without any detectable antitoxin in the circulation may be killed by the injection of 1/100 c.cm. of toxin of which 10 c.cm. would be tolerated easily by horses containing over one-tenth of a unit of antitoxin per c.cm. of blood. It is generally stated that non-immune horses are far more susceptible than guinea-pigs and rabbits. It appears from the little work that has been done, taking into consideration the possibility of natural immunity, that most animals, if they contain no circulating antitoxin, are at least as susceptible as the rabbit and guinea-pig. The chief exceptions are the rat and the mouse. Cobbett (1899<sup>2</sup>) showed that for a rat of 100 gm. it was necessary to use about 600 to 700 times as much diphtheria toxin as would be required to kill a guinea-pig of 250 gm.; that is to say, a rat can tolerate 1,500 to 1,700 times more toxin than can a guinea-pig of equal weight. He concluded

that the comparative insusceptibility of the rat was not due to antitoxic action of the blood-serum, and therefore it differs from the acquired immunity of animals immunized to these poisons. Pettit (1913 and 1914) has studied the resistance of the rat to diphtheria toxin and found that a rat of 100 gm. could tolerate 10 to 15 times the m.l.d. for a rabbit or more than 100 times the m.l.d. for a guinea-pig or a pigeon. Coca, Russell and Baughman (1921) state that 'the rat is not absolutely immune to diphtheria toxin. Although it usually survives the injection of 1,000 m.l.d. (for the guinea-pig), it regularly succumbs to 4,000 such units'. They suggest that toxin is less rapidly absorbed by the cells of the rat than by those of the guinea-pig. Further, they show that repeated injection of toxin into rats stimulates the production of antitoxin. Ramon, Noureddine and Erber (1928) found that rats of 150 gm. were killed by 700 to 1,000 guinea-pig m.l.d. Glenny and Allen (1922<sup>1</sup>) find that 'the intravenous m.l.d. for mice is 60 times, and the intramuscular m.l.d. for mice 100 times, the guinea-pig subcutaneous m.l.d.'. They further show that antitoxin has the same neutralizing power for toxin in mice as in guinea-pigs. Jungeblut (1927) states that mice of 24 to 30 gm. in weight are killed by 75 to 100 guinea-pig m.l.d. injected intravenously, or by 200 m.l.d. injected intraperitoneally.

Strubell (1910) compared hedgehogs with guinea-pigs in their ability to tolerate diphtheria and tetanus toxins, and he found that the guinea-pig was 8 times more sensitive to diphtheria and nearly 15 times more sensitive to tetanus than the hedgehog for equivalent weights.

#### *Relationship between m.l.d. and m.r.d.*

The relation between m.l.d. and m.r.d. has been variously expressed at different times, depending upon the end point that has been taken to denote the minimal reaction. Römer (1909) states that 1/250 m.l.d. causes redness followed by necrosis on the third day, and 1/500 m.l.d. causes slight redness on the first and second day, disappearing on the third day. Glenny and Allen (1921) affirm that about 1/500th of a fatal dose of toxin will produce a definite intracutaneous reaction. Improved technique has made it possible to detect reactions caused by smaller amounts of toxin. Glenny, Pope and Waddington (1925) give the approximate relationships as 1 m.l.d. = 1,000 m.r.d., but more recent tests have shown that 1/2,000th m.l.d. produces a faint reaction 4 or 5 mm. in diameter, visible on a well-shaved healthy guinea-pig on the second day after injection. Brokman and Sparrow (1925) say that 1/800th m.l.d. will produce an intracutaneous reaction in the majority of guinea-pigs, but that some 5 per cent. require 1/300th m.l.d.

For rough tests, as a preliminary guide to toxicity, dark-skinned guinea-pigs may be used, but for the determination of the m.r.d. by the intracutaneous method of Römer (1909), it is essential to use white or very pale-skinned guinea-pigs, preferably between 300 and 400 gm., and to make all injections on the flanks. Smaller animals may be used but

reactions tend to be less distinct ; the skin of larger animals is often scratched and rendered unsuitable. Coca, Russell and Baughman (1921) recommend guinea-pigs weighing over 400 gm. ; however, they inject into the skin of the abdomen.

The intracutaneous method forms a most convenient guide to the progress of modification of a toxin into toxoid. If 0·2 c.cm. of the undiluted material fails to cause a specific skin reaction, 5·0 c.cm. will not produce symptoms in a guinea-pig when injected subcutaneously.

#### TESTS FOR COMBINING POWER.

##### *Subcutaneous Method.*

In the subcutaneous test for combining power there are two convenient end points : the death of a guinea-pig within a reasonable time, five days, and the border line between presence and absence of local oedema. These two end points of Ehrlich give the two limiting values of a toxin, the L+ and the Lo doses.

The Lo dose of toxin mixed with one unit of antitoxin and injected subcutaneously into a guinea-pig causes no oedema, but there may be toxin free in too small a quantity to cause any detectable symptoms. For this reason Ehrlich (1906) states that this measurement should be determined so that in an Lo mixture 'all the constituents of the poison are completely neutralized, so that not only the single but also high multiples of this can be injected into guinea-pigs without causing a trace of a local or general reaction'. The difference between the Lo dose determined against one unit of antitoxin and that determined by injecting large multiple doses may be as great as 5 per cent. The true multiple Lo dose corresponds very closely with the Lr dose.

Antitoxin is tested by titration against the L+ dose of toxin which has been fixed by testing against one unit of standard antitoxin. In practice, a toxin is chosen that has been kept for a year or more under toluol until stable. A weak toxin should be used having an L+ dose between 0·5 and 1·0 c.cm. for convenience in measurement. Both Rosenau (1905) and Dean (1908) state that the difference between the Lo and L+ should not be more than 15 m.l.d. If a toxin is used with the zone between Lo and L+ (the 'differential region' of Ehrlich) extending over a wider range, the end point is less sharp. The greater the difference between the Lo and L+ dose, the greater will be the difference in the amount of antitoxin necessary to delay death from the third to beyond the fourth day and the less exact will the end point become. A very few tests in series are sufficient to establish a test-dose of the toxin and the unit of antitoxin to an accuracy of 5 per cent.

In order to ensure that the full amounts of toxin and antitoxin are injected into a guinea-pig Rosenau (1905) measures the test-dose direct into a syringe. A separate syringe is provided for each test to be made ; the toxin is first measured into the barrel, then the diluted serum. The two are thoroughly mixed by agitating the syringe and the mixture is

allowed to stand at room temperature in diffused light for one hour and is then injected into the guinea-pig. Next, with the needle left *in situ*, the bulb is removed from the syringe and enough salt solution to bring the total volume to 4 c.cm. is quickly run into the barrel, washing down the sides; this in turn is injected into the animal. Such extreme precaution seems to the writer, however, hardly necessary. Rosenau (1905) insists upon the necessity of using guinea-pigs under standard conditions and of approximately the same age. They should be bred from reliable stock and fed and caged so as to ensure vigorous animals. After inoculation the guinea-pigs are kept two in a cage and not disturbed. Keeping many animals together in a pen may give irregular results. Examining the guinea-pigs too often or too roughly must also be avoided.

#### *Intracutaneous Method.*

The intracutaneous method introduced by Römer and Sames (1909) is of far greater value to the immunologist than any of the subcutaneous methods. An average of 15 times as many titrations can be made with the same expenditure of guinea-pigs; all the test animals survive and can be used for other purposes; the results of tests are obtained in two days instead of four; the accuracy of testing is at least as great as with the subcutaneous method. The author suggests that this method deserves serious consideration by those responsible for the accurate preparation of standards. On theoretical grounds a more nearly total neutralization test is preferable to one in which a considerable amount of toxin is left unneutralized. The use of a nil end point by subcutaneous injection (Lo) is difficult, but by intracutaneous injection traces of free toxin are easily detected.

The Lr dose of test toxin is determined by preparing a number of mixtures containing different amounts of undiluted toxin and one unit of antitoxin and testing for excess of toxin by first injecting 0·2 c.cm. of the mixtures diluted 1 in 100 and then injecting undiluted only such mixtures as have failed to produce a reaction when diluted. Accurate standardization of sera is similarly done by testing a series of mixtures of the unknown antitoxin with one Lr dose of toxin.

For general purposes, subsidiary units, such as Lr/500, are used for titrating toxin and antitoxin. The subsidiary unit is of considerable value for estimating the *in-vivo* combining power of fresh toxins when it is not necessary to determine the strength of the toxin to an exact degree. The Lr dose of a fresh toxin is about 450 times the Lr/500 dose. It is obvious that slightly more than the Lr dose must be added to one unit of antitoxin in order that 1/500th of the mixture should contain one m.r.d. of free toxin. The smaller unit should not be used in testing old toxins or any unusual modifications of toxin that may contain much toxoid, because the number of reacting doses in the test volume may be too small. For titrating antitoxin at this level, the test toxin is diluted to contain 10 Lr/500 doses per c.cm. and mixtures are made up to 2·0 c.cm. and

0·2 c.cm. injected. When only small amounts of serum are available for test, titrations may be made at the 1*r*/2,000 level. When serum is tested for antitoxic values below 0·1 unit, titrations are made by mixing equal parts of undiluted serum and known dilutions of toxin. If a fresh toxin is used, it is possible to detect the presence or absence of 1/50,000 unit of antitoxin in 0·1 c.cm. serum.

*Intracutaneous tests in rabbits.* Fraser and Wigham (1924) recommend the use of rabbits in place of guinea-pigs for the intracutaneous test. They state that the skin of rabbits appears to be much more sensitive to diphtheria toxin than that of guinea-pigs. The variation between the reactions produced in individual rabbits has been slight. One may rapidly test forty mixtures on one animal, including three or more control mixtures to serve as a basis for comparison. The advantages of using rabbits in place of guinea-pigs are economy of time and animals, greater facility in reading the reaction and uniform basis of comparison for many tests on the same animal.

*Titration of human serum.* The intracutaneous method of titration has been used for many years to control the Schick test when doubtful readings have been made. Kellogg (1922) has suggested that the Schick test should be replaced by a single titration for the presence or absence of 1/30 unit of antitoxin per c.cm. of blood. In the author's experience it is always preferable to test any sample at two different levels on the same guinea-pig; the gradation in reactions produced helps to fix the limits at which closer tests should be made, occasional non-specific reactions are more readily detected, and when many tests are made upon the same animal any possible errors due to badly spaced injections are more easily avoided. It should be pointed out here that there is no justification for the use of the term 'Kellogg test'. The intracutaneous method of testing the antitoxic content of human serum apart from, or in conjunction with, the Schick test has been in almost universal use for many years.

*The Schick test* is a special application of a test for combining power by intradermic injection. The injection of a small dose of toxin into the skin of man produces a similar reaction to that obtained in guinea-pigs and other animals; this reaction is prevented if sufficient antitoxin is injected at the same time or if antitoxin is already present in the test animal. It has been pointed out by Glenny and Allen (1922<sup>2</sup>) and by Kellogg (1922) that the original definition of the Schick dose as 1/50th m.l.d. of a matured toxin is insufficient. Glenny and Waddington (1929) prepared dilutions of three different matured toxins each containing 1/50th m.l.d. per c.cm. The antitoxic levels of immunity detected in guinea-pigs injected intracutaneously by these three dilutions were 1/250th, 1/100th and 1/50th of a unit per c.cm. respectively. It is necessary, therefore, to include in the definition a reference to the combining power of the toxin. Table II shows a typical routine titration of a dilution of toxin prepared for Schick testing.

TABLE II.

Showing a Typical Routine Titration of a Dilution of Toxin prepared for Schick Testing.

*Test for m.r.d.*

Dilution.	Measurements.	Diameter of Reaction produced by the Intracutaneous Injection of 0.2 c.cm.
1/10	0.5 toxin dilution + 4.5 saline.	
1/15	1.0 of 1/10th toxin dilution + 0.5 saline.	15 mm. Intense.
1/20	1.0 " " " + 1.0 "	14 mm. Intense.
1/25	1.0 " " " + 1.5 "	14 mm. Intense.
1/30	1.0 " " " + 2.0 "	8 mm. Moderate.
1/40	1.0 " " " + 3.0 "	3 mm. Faint.
1/50	1.0 " " " + 4.0 "	Nil.

*Test for Combining Power.*

Toxin.	Dilution of Antitoxin containing 1/200th Unit per c.cm.	Diameter of Reaction produced by the Intracutaneous Injection of 0.2 c.cm.
1.0 c.cm.	1.0 c.cm.	3 mm. Very faint.
1.0	0.8	12 mm. Faint.
1.0	0.6	15 mm. Intense.
1.0	0.5	16 mm. Intense.

*Method of testing total combining power of toxoid.* When enough antitoxin is added to a fresh toxin to neutralize all specific toxicity almost all the toxoid present is also in combination ; the Lr is therefore almost an exact index of the total amount of toxin and toxoid present. Old toxins or partially modified toxins contain toxoid in considerable excess of toxin ; upon addition of antitoxin all specific toxicity may be neutralized although a considerable amount of toxoid still remains uncombined ; the Lr dose is not a correct index of total combining power of such old or modified toxins. If a toxin is completely modified into toxoid, no intracutaneous reaction is produced and therefore no Lr measurement can be made. Some indication of combining power can be obtained by determining the Lr dose of a mixture of a modified toxin with fresh toxin of known strength,

but the relative avidity of the toxin and toxoid may lead to discrepant results if the fresh toxin combines more easily with antitoxin than the modified toxoid. A more satisfactory method is to add antitoxin to the modified toxin, and after leaving a sufficient time for combination to take place, to test the mixture for uncombined antitoxin. Glenny, Hopkins and Pope (1924) have pointed out some of the difficulties of this method of titration. It should only be resorted to if the flocculation method fails, and should be regarded as a definite experimental procedure rather than a routine method.

*In-vitro Method.*

Under suitable conditions, the combination of an antigen with its antibody is accompanied by the formation of a flocculent precipitate. In 1902, Danysz first showed this for ricin and antiricin; in 1909, Calmette and Massol found that a similar precipitate was formed between venom and antivenom; and different workers have since shown that it occurs between most bacterial toxins and their antitoxins. Ramon (1922<sup>1</sup>) has studied diphtheria toxin and antitoxin in detail. Glenny and Okell (1924) reported a second zone of flocculation, and suggested that it was probably due to the reaction between bacterial protein and antiprotein and not between toxin and antitoxin. H. Schmidt (1926) and Moloney and Weld (1925<sup>1</sup>) confirmed this by showing that rabbit-serum, obtained from animals injected with washed diphtheria bacilli, flocculated with diphtheria toxin, but did not remove its binding power for antitoxin. With tetanus toxin and antitoxin, it may be due to the presence of an additional antigen, lysin, that a further zone of flocculation occurs. Kalic (1928) observed 2 zones of flocculation in 53 tetanus toxins examined by him, and 3 zones in 3 of these toxins. Further study is needed to show what substances are removed in each precipitate. An interesting comparison can be made between the toxin flocculation reaction and the precipitin reaction. Goldsworthy (1928), when testing 25 samples of horse-sera against rabbit antihorse-serum, found 7 samples with 3 zones and 12 samples with 2 zones. The multiplicity of antigens appears to cause the formation of different zones of precipitation.

Bronfenbrenner and Reichert (1926) doubt the specificity of the flocculation reaction. Using serum obtained from an animal immunized against autolysed bacilli, they found that botulinus toxin was precipitated, but this did not occur when antitoxic serum was used. They suggest that uniformity in toxin production in all laboratories would lead to a constant relation between specific diphtheria toxin content and bacterial protein in the culture filtrate. The concentrations of antitoxin and of antibacterial body in the serum produced by immunization with these filtrates, therefore, bear sufficiently fixed relations to each other for comparable results to be obtained by both *in-vivo* and *in-vitro* titration. The following facts should remove all such doubts as to the specificity of the flocculation reaction.

Reasonable uniformity between in-vivo and in-vitro tests is obtained with toxins prepared from strains other than Park 8, with many types of media and with great variation in length of growth. Where two zones occur in the flocculation reaction, one precipitate is antigenic while the second is not antigenic. Upon removing the precipitate formed after the addition of agglutinating serum, toxin will still flocculate with the antitoxin, and antitoxin can be recovered from the toxin-antitoxin precipitate.

The flocculation test of Ramon (1922<sup>1</sup>) affords a simple and reliable method of titrating toxin and antitoxin without the use of experimental animals. Ramon employed 20 c.cm. of a high-grade toxin in each tube of his test, and added to a series of such tubes varying amounts of undiluted antitoxic serum. Later workers have found 5 c.cm. or even 1 c.cm. of toxin to be sufficient. If the tubes are left, flocculation takes place in certain of them, and it usually appears first in the mixture which is most nearly neutral and spreads subsequently as a zone on either side of this. The mixtures with a marked excess of either toxin or antitoxin show no flocculation. The first precipitate to appear in the mixture Ramon called the 'précipite indicateur', as it is from the quantities of toxin and antitoxin in this mixture that the values are calculated. Ramon has suggested the term 'antigenic unit' for the amount of toxin equivalent to one unit of antitoxin determined by the flocculation test. The use of the term Lf suggested by Glenny and Okell (1924) is preferable, as it is in accord with the already existing and accepted terms Lo, L+ and Lr, used in defining the combining properties of individual toxins.

*Rate of flocculation.* The essential feature of the in-vitro method of testing toxin and antitoxin is the speed at which mixtures flocculate. The 'précipite indicateur' is the precipitate that forms most rapidly, and there are many suggestions, not yet fully supported experimentally, that speed of flocculation is a measure of avidity. It is important, therefore, to consider all factors influencing the rate of flocculation in individual tests of a series and in one series compared with another.

*Relative proportions of toxin and antitoxin.* The rate at which any mixture of a given toxin and antitoxin flocculates under fixed physical conditions depends upon the balance between the antigen (toxin plus toxoid) and antibody. Curve 1 on Chart I shows the relationship between time of flocculation and composition of mixture determined by Glenny and Wallace (1925).

*Strength of toxin.* Under similar conditions the rate of flocculation of balanced mixtures of different batches of toxin with a given antitoxin depends upon the strength of the toxins, provided that they are tested when relatively fresh and have been prepared in a similar manner. In a series of toxins quoted by Glenny and Wallace (1925) all batches between 4 and 8 Lf units per c.cm. flocculated in 75 to 100 minutes, those between 8 and 12 Lf units in 40 to 50 minutes, those between 12 and 18 Lf units in 20 to 30 minutes, and those between 18 and 28 Lf units in 10 to 15 minutes.

*Quality of toxin used.* Old toxins flocculate more slowly than those freshly prepared. Ramon (1923) mentions a toxin kept for four months at 38° C. without alteration in flocculating value, but with increased flocculating time. According to Feierabend (1925) toxins have a qualitative value dependent upon the time of flocculation. He suggests that these differences may depend upon the avidity of toxin for antitoxin. Modification of toxin by formaldehyde increases the time of flocculation; a toxin which when untreated flocculated in 30 minutes, after 10 days incubation with 0·2 per cent. formaldehyde flocculated in 2 hours. Concentrated toxins vary greatly in their flocculation rate.

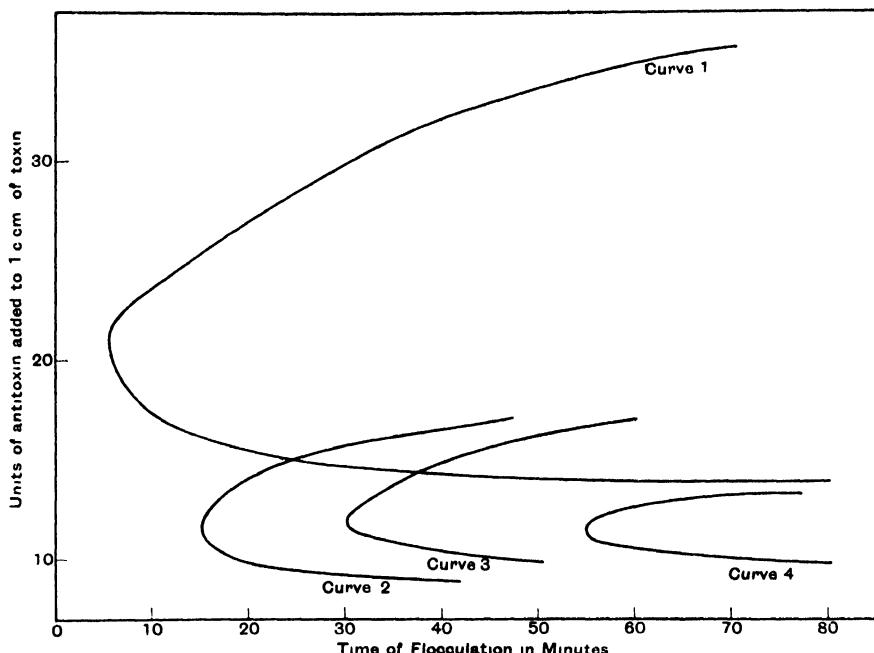


CHART I. Flocculating times in minutes of mixtures containing 1.0 c.cm. of toxin and different amounts of antitoxin. Curve 1: Toxin containing 21 Lf units per c.cm.; mixtures kept at 50° C. Curves 2, 3 and 4: Toxin containing 12 Lf units per c.cm.; mixtures kept at 50, 35 and 25° C.

*Quality of antitoxin.* Ramon (1922<sup>a</sup>) stated that sera of the same value flocculated in different times and suggested that the differences were possibly due to physical or chemical differences or to differences in specific affinity. Madsen and Schmidt (1926) stated that the speed of flocculation is relatively constant for the same horse, and produced evidence to show that speed of flocculation may be some measure of avidity (see p. 150). Sera that give a higher value when tested *in vivo* than *in vitro* usually flocculate very rapidly, while those with the reverse ratio are slow. Modified antitoxin (Glenny, 1913) which has poor affinity for toxin gives a higher value *in vitro* than *in vivo* and is slow to flocculate. It would appear, therefore, that there may be some connection between

avidity and rate of flocculation, but there can be no exact correlation unless other chemical and physical differences in sera exert a similar effect upon speed of visible flocculation and upon rate of combination with toxin. Schmidt (1928) produced further evidence of the connection between avidity and rate of flocculation of both diphtheria and tetanus antitoxin.

The relative constancy of rate of flocculation of successive samples of serum from the same horse is shown by the following figures : 10 samples of sera from one horse flocculated in times varying from 14 to 35 minutes, while 8 samples from another horse all flocculated in from 43 to 80 minutes when tested under the same condition against the same toxin. According to Locke, Main and Miller (1927) there appears, however, to be some tendency for more rapid flocculation in the very early stages of immunization.

The rate of flocculation becomes slower as serum ages. Serum kept at 37° C. soon loses its power to flocculate and will inhibit flocculation in fresh serum when blended in equal parts, although the serum still shows considerable protective value *in vivo*. Ramon (1922<sup>2</sup>) states that pure pseudo-globulin, although it may be highly antitoxic *in vivo*, forms no precipitate, but on addition of euglobulin flocculation appears rapidly. Renaux (1924) compared the rate of flocculation of various blends of fresh antitoxin and the same antitoxin heated for 1 hour at 56° C. and he found that 100 per cent. fresh serum without heated serum flocculated in 60 minutes, 75 per cent. fresh serum and 25 per cent. heated serum flocculated in 90 minutes, 50 per cent. fresh serum and 50 per cent. heated serum flocculated in 120 minutes, 25 per cent. fresh serum and 75 per cent. heated serum flocculated in 160 minutes. No fresh serum but 100 per cent. heated serum flocculated in 330 minutes.

*Concentration of mixtures.* Dilution delays the rate at which mixtures flocculate. It is frequently difficult to determine the indicating tube in a series of mixtures that flocculate rapidly ; dilution may then be used to advantage. The following example is recorded by Glenny and Wallace (1925). A serum was chosen with so rapid a 'spread' of flocculation that all mixtures containing between 13·5 and 19·0 units per c.cm. of a toxin appeared to flocculate simultaneously when incubated at 50° C. By diluting the mixture five- and tenfold, flocculation was delayed from 12 minutes to 94 and 150 minutes, and the 'spread' occurring within 5 minutes was reduced from between 11·7 and 22·0 units to between 13·2 and 17·2 and 13·0 to 14·6 units. In these experiments, the antitoxin was added in increasing amounts to 1 c.cm. of undiluted toxin, to 5·0 c.cm. of a fivefold dilution, and to 10·0 c.cm. of a tenfold dilution, but only 1·0 c.cm. of each mixture was incubated. Pope (1927) has shown that the local concentration of material that occurs when toxin-antitoxin mixtures are frozen causes flocculation to occur over the same range of tubes as that apparent when mixtures are heated.

*Hydrogen-ion concentration.* Bayne-Jones (1924) states that the results of titrations at different pH levels beyond the range 7·0 to 8·4 were

irregular and of no significance, because non-specific precipitates were formed obscuring the flocculation. Watson and Langstaff (1926) found that concentrated toxoid between the range pH 6·0 to 8·0 flocculated in the same time, at pH of 9·0 or 10·0 flocculation was delayed, at pH 5·0 flocculation was accelerated.

*Salt content.* Small differences in salt content of toxin do not appear to affect rate of flocculation to any great extent. The following figures show the effect of relatively large additions of sodium chloride :

		<i>Toxin 'A'.</i>	<i>Toxin 'B'.</i>
		<i>Rate of Flocculation.</i>	<i>Rate of Flocculation.</i>
Control .. ..		30 to 40 minutes.	45 to 55 minutes.
+ 1 per cent. NaCl .. ..		40 „ 45 „	60 „ 65 „
+ 2 „ ..		50 „ 60 „	70 „ 85 „
+ 5 „ ..		80 „ 90 „	120 „ 140 „

Schmidt (1928<sup>1</sup>) found that in equal molecular concentrations sodium bromide had a greater retarding influence than sodium chloride ; sodium iodide had a still greater influence and manganese chloride delayed flocculation more than any of the other salts tested by him.

*Temperature.* Curves 2, 3, 4, on Chart I (p. 117) show the rates of flocculation of a series of mixtures kept at temperatures of 50°, 35° and 25° C. The times of flocculation are 15 minutes, 30 minutes and 55 minutes respectively. From the shape of the curve it will be seen that the rate of spread of flocculation to adjacent mixtures in the series is delayed at the lower temperatures and the ease with which readings can be made is correspondingly greater. Times of flocculation quoted by Glenny and Okell (1924) for a certain mixture are : 15 minutes at 50° C., 23 minutes at 37°, 110 minutes at 15°, and several days at 0°. Bayne-Jones (1924) found that a certain mixture flocculated in 12 minutes at 55° C., in 30 minutes at 37°, in 60 minutes at 22°, and in 24 hours at 5° to 10°.

Use may be made of delayed flocculation at low temperatures when it is necessary to obtain accurate readings with mixtures showing a wide 'spread'. If mixtures are made late in the day it is often convenient to place them in an ice-chest over-night and to transfer them to a warm bath next morning.

*Convection currents.* In a series of mixtures at narrow limits discrepant readings always occur unless considerable care is taken to ensure uniformity of conditions in all tubes of the series. Glenny and Wallace (1925) give the following example. Equal volumes of the same mixture in tubes of different width, 0·5 cm. and 0·75 cm. in diameter, flocculated in 40 minutes and 47 minutes respectively under identical conditions with one-third immersed, and in 54 minutes and 74 minutes respectively when fully immersed ; 2·0 c.cm. and 1·0 c.cm. of the same mixture in tubes of the same diameter flocculated in 42 minutes and 47 minutes side by side in the bath, the tubes being equally, and the columns of liquid unequally, immersed. If, however, uniformity of heating and of convection

currents is ensured by totally immersing the tubes, the volume of mixture, provided there is sufficient to give a visible precipitate, does not appear to influence the rate of flocculation ; 5·0 c.cm. and 1·0 c.cm. of the same mixture, completely immersed, both flocculated in 40 minutes ; it is, however, difficult to ensure a simultaneous rise of temperature in very unequal volumes of liquid in the same bath.

*Method of blending.* It may on occasion happen that the serum or toxin to be titrated is of absolutely unknown value, and no flocculation may occur unless tests are made upon a very long range of mixtures. A contrast between the in-vitro and in-vivo tests lies in the fact that from the injection of one mixture into an animal it can be decided whether the serum is above or below a certain value, whereas in the Ramon test no indication is given whether the value lies above or below those tested if no flocculation occurs. Glenny and Okell (1924) suggest that an unknown should be blended with equal parts of a known toxin or antitoxin to put a limit on the low value to be detected. For example, if an unknown serum is blended with one containing 400 units per c.cm. the resulting mixture must contain 200 units or more and a short series at wide range, testing for say 200, 300, 500, 800 and 1,200 units per c.cm., will give an indication of the limits of values of the unknown serum ; once these limits are known the direct method of titration can be employed. Renaux (1924) suggested the method of blending in the titration of old or altered serum. He showed that a mixture of equal parts of a fresh antitoxin with a non-flocculating serum of known in-vivo value behaves as a slow flocculating serum of a mean value between the two. Bayne-Jones (1924) suggests the method of blending for the titration of purified pseudo-globulin.

*Absence of flocculation.* Flocculation depends upon the amount and relative proportions of toxin and antitoxin and upon some chemical or physical properties of the specific antigen and antibody or of substances in the surrounding medium. If the amount of toxin and antitoxin falls much below 1 Lf unit and 1 in-vitro unit respectively, it is difficult to detect flocculation. Small amounts of toxin and antitoxin must be titrated by animal methods which will detect 1/1,000th of the minimum detectable by the in-vitro method. A given series of mixtures may not include one in which the components are present in sufficiently balanced proportions to cause flocculation, if the range is set at too wide limits or if tests are made at too high or too low a level. The method of blending should then be adopted. Direct tests may be applied at closer limits round the value detected by the blend method. Failure to flocculate by the direct method does not imply an absence of any combining value unless the blend method has also shown such absence. An old serum may fail to flocculate unless some fresh antitoxin is added.

A toxin may be so modified that no flocculation will occur, although it contains many Lf units, and then these can only be demonstrated by blending the modified toxin with fresh toxin. Absence of flocculation does not mean absence of toxin or toxoid. An Lf value with an infinite

flocculating time can be assigned to such a toxin. Glenny and Waddington (1928) have shown that toxoid with an excess of formaldehyde fails to flocculate.

*Non-specific flocculation.* According to Glenny and Wallace (1925) certain sera are found to possess two zones of flocculation, one specific zone corresponding to the in-vitro value and the non-specific zone varying independently of the Lf value for different batches of toxin used for titration. Two zones were seen in 17 successive samples of serum taken from the same horse and one sample showed two zones when mixed with 20 out of 24 samples of toxin. Some batches of diphtheria toxin have shown non-specific flocculation with both antitoxic and normal horse-serum.

*Relation between in-vivo and in-vitro testing.* In the present state of our knowledge it is assumed (although the assumption is as yet incapable of proof) that in a mixture that flocculates first in a series the toxin-plus-toxoid content is exactly equivalent to the antitoxin present. Such a mixture produces no apparent symptoms in animals, but with an average sample of fresh toxin and antitoxin, 10 per cent. less antitoxin is present in an Lr mixture. As toxins age or are chemically modified into toxoid, the Lf/Lr decreases until, when there is no longer any specific toxin present, the Lr dose is infinite and the ratio 0. Typical examples of the ratio between the Lo and Lf of toxins depending upon the number of m.l.d. per Lf are given later in Tables III and IV (p. 144). The flocculation test has the advantage over any animal titration not only in the ease and rapidity with which titrations are made, but also because it is a direct measure of total binding capacity, irrespective of whether the combining substance is toxin or toxoid. Before Ramon's introduction of the test, the extent to which destruction had occurred when toxin was modified into toxoid could only be measured by the laborious and inaccurate process of first mixing with antitoxin and then titrating the uncombined antitoxin against a known toxin.

When the Lf/Lr ratio of any fresh toxin is determined against many samples of antitoxin it is found that although with most samples the value of the ratio falls between 0·80 and 0·95, in some exceptional cases the ratio may be lower than 0·4 or higher than 2·0. For this reason the definition of the Lf unit is referred to 'a certain chosen antitoxin', and it is necessary to refer to different in-vivo and in-vitro unit values of certain antitoxins. Glenny and Wallace (1925) obtained consistent Lf values for a number of different toxins tested against one in-vitro unit of three samples of antitoxin equal to 1·0, 1·3 and 2·36 Ehrlich units respectively. Moloney and Weld (1922-5), and Sordelli and Serpa (1925) have found similar discrepancies between animal tests and the flocculation method. Many workers claim complete agreement between the two methods of titrating diphtheria toxin and antitoxin, but have found discrepancies in similar tetanus titrations. Ramon (1927) states that he has very occasionally observed some slight abnormal flocculation, as some horses produce 'un sérum pathologique'.

Glenny, Pope, Waddington and Wallace (1925<sup>1</sup>) have shown that the 'serum ratio'  $\frac{\text{in-vivo value}}{\text{in-vitro value}}$  is often constant for successive samples of serum from the same horse, but usually 'there is a general tendency for the ratio to be higher in the earlier tests and to become constant as immunization proceeds'. Unusual serum ratios appear more frequently among sera of relatively low titre; if the ratio  $\frac{\text{in-vivo value}}{\text{in-vitro value}}$  is high the serum usually flocculates rapidly; if the serum ratio is low flocculation is slow and the serum corresponds to the modified antitoxin of Glenny (1913). The serum ratio is unaltered by concentration by the ammonium sulphate process. The fact that it has been demonstrated without doubt that great discrepancies may sometimes occur between in-vitro and in-vivo titrations makes it imperative that confirmatory animal tests should be made upon antitoxin intended for therapeutic use.

*Ring precipitation.* Nicolle, Césari and Debains (1920) appear to have been the first workers to obtain a layer neutralization effect between toxin and antitoxin. The toxic material was mixed with gelatin and allowed to set in 1 c.cm. quantities. On to these solidified solutions were then run 1 c.cm. amounts of varying dilutions of antitoxin, and at the junction there appeared a bluish-white ring after 2 hours at laboratory temperature, at the maximum point of neutralization. Hoen, Tschertkow and Zipp (1927) have used a method of estimating the value of antitoxin or toxin by means of a ring precipitation between the two. Various dilutions of the toxin are layered on the serum diluted with 5 per cent. saline containing 20 per cent. normal horse-serum. The tube in which the ring is formed is stated to correspond to the Ramon flocculation point and the term Lp is given to it. Hoen and his co-workers claim that in certain circumstances their method is a truer index of the combining power than the flocculation test of Ramon. This claim does not seem to be justified, but it appears possible that under circumstances where flocculation is difficult to obtain, the ring precipitation method might be successfully applied.

#### The Action of Chemical and Physical Agents upon Toxin.

The action of chemical and physical agents upon toxin depends largely upon the type of medium used in the preparation of the toxin and upon the method of its preservation; thus, Glenny and Walpole (1915) failed to produce a precipitate upon the addition of acetic acid to crude diphtheria toxin prepared from a peptone beef infusion medium, but Watson and Wallace (1924<sup>1</sup>) were successful with toxins prepared from other types of medium. According to different authors the pH at which toxin precipitates varies, and depends upon the type of toxin used and upon whether the toxin has been modified by the action of formaldehyde. This is, of course, what would be expected, since all the precipitates so far worked with are probably protein compounds with only minute traces

of pure toxin. The behaviour of toxin is considerably altered by the presence or absence of phenolic preservative. Toxin containing phenol is far less heat-stable than toxin without preservative, and is more sensitive to changes in pH. It is important, therefore, to bear in mind that in all work on toxin, consideration should be given to the presence of phenol, tricresol or other antiseptic.

There are three effects that may be produced by the action of any chemical or physical agent upon toxin : (a) modification of toxin into toxoid ; (b) precipitation of some or all of the toxin or toxoid ; (c) destruction of toxin or toxoid. In addition to these effects, which can be quantitatively measured, there may be qualitative differences in the toxoid formed as well as an alteration in the surrounding medium. It is known that different preparations containing toxoid vary in their antigenic efficiency, but as neither toxin nor toxoid have been isolated in the pure state, it is at present unknown whether these differences are due to (a) different types of specific toxoid, or (b) differences in the substances on which the toxoid is adsorbed or with which it may be combined, or, (c) differences in the surrounding medium. Much of the work in the past is of little value because of the failure to recognize the three possibilities of modification, precipitation or destruction. It was sufficient to know as the result of early work that such chemicals as iodine trichloride 'attenuated' toxin, and that such attenuated toxin would produce a basal immunity in horses : or that toxin preserved with formaldehyde could still be used for immunization and hyperimmunization although the toxicity gradually disappeared. The flocculation test of Ramon and the intracutaneous test of Römer have greatly increased the facilities for tracing the changes which occur. Of these the most important is the modification of toxin into toxoid.

#### TOXOID FORMATION.

The number of fatal doses with which one unit of antitoxin can combine, appears to vary with different batches of toxin. When Ehrlich first observed this apparent difference, he investigated many batches of toxin, some freshly prepared, some precipitated with ammonium sulphate and others that had been kept for some time, and he found that the number of m.l.d. contained in L+ doses varied between 15 and 160 for the various preparations he tested. He concluded that 'these results can only be explained by assuming that the diphtheria bouillon, in addition to the toxins, contained other non-toxic substances which were able to combine with antitoxin just like the diphtheria toxin'. He found that 'bouillon on standing can preserve its neutralizing property intact, and often actually does so, while the toxicity is considerably decreased'. This altered toxin that was no longer toxic but combined with antitoxin, he termed 'toxoid'. Later, he assumed the existence of many types of toxoid and of toxone. Evidence is submitted later (see p. 137) that there is no need to suppose more than one kind of toxoid. For the present, therefore, we will refer

only to toxoid in a general sense to cover all these possible types of modified toxin, and define toxoid as that altered form of diphtheria toxin which no longer causes any symptoms when injected into animals, but is still capable of combining with antitoxin. Not only does it combine with antitoxin, but it also acts as an antigen and stimulates the production of antitoxin in the blood of the animal into which it is injected, and is thus a harmless material which can be used in immunization. It is this property of diphtheria toxoid that makes its study of such importance.

*The Action of Formaldehyde on Toxin.*

The most reliable method of modifying toxin to toxoid is by the action of formaldehyde at a temperature of 37° C. Formaldehyde was used in the early days of immunology, together with other chemicals such as iodine trichloride, for the purpose of attenuating diphtheria or tetanus toxin used in the early stages of immunization of horses. Recognition of the variation in the normal antitoxic content of the blood of horses at a later date rendered this attenuation unnecessary. At a still later date, the introduction of active measures for human immunization revived interest in the subject, but investigation was carried on at considerable disadvantage until the introduction of the flocculation test of Ramon. The use of formaldehyde to modify tetanus toxin was reported by Löwenstein (1908), v. Eisler and Löwenstein (1911) and by Monteiro (1921). Similarly modified diphtheria toxin was described by Glenny and Südmersen (1921), by Glenny and Hopkins (1923), and by Ramon (1923<sup>1</sup>). The attenuated toxin first used for horse immunization was produced by the addition of 0·1 per cent. formaldehyde (approximately 0·25 per cent. commercial formalin), and the toxin was left at room temperature. A rapid reduction of toxicity occurred, sufficient to render the toxin harmless for horse immunization. Further loss in toxicity was very slow and some years elapsed before such toxin was so completely modified that the injection of 5 c.cm. produced no symptoms in guinea-pigs. The influence of exposure to higher temperatures for the rapid reduction in toxicity is shown by the following figures (Glenny, Hopkins and Pope, 1924). To a certain toxin, 0·3 per cent. formaldehyde was added and different samples after 24 hours exposure to temperatures of 30°, 33°, 36° and 39° C. were found to contain 1,250, 500, 250 and 25 m.r.d. per c.cm. respectively. The following figures from the same authors show the effect of varying the amount of formaldehyde added and exposing the mixture for 24 hours to a temperature of 37° C. :

0·01 per cent. formaldehyde reduced the toxicity to  $\frac{1}{2}$  the original

0·03	"	"	"	"	"	$\frac{1}{3}$	"
------	---	---	---	---	---	---------------	---

0·1	"	"	"	"	"	1/20	"
-----	---	---	---	---	---	------	---

0·2	"	"	"	"	"	1/100	"
-----	---	---	---	---	---	-------	---

0·3	"	"	"	"	"	1/400	"
-----	---	---	---	---	---	-------	---

0·4	"	"	"	"	"	1/10,000	"
-----	---	---	---	---	---	----------	---

0·5	"	"	"	"	"	a non-toxic state.	
-----	---	---	---	---	---	--------------------	--

Too high concentration of formaldehyde is not advisable and a longer exposure at a lower concentration is preferable. The amount of formaldehyde necessary and the length of exposure depend upon the constitution of the toxin to be modified. Some of the formaldehyde is absorbed by amino-acids, which vary considerably in amount in different batches of toxin. It appears possible that the modification of toxin by formaldehyde is of a different nature from the combination of formaldehyde with free amino groups, for the modification of toxin is a much slower process ; it is, however, true that the Van Slyke nitrogen determination of toxin to which formaldehyde has been added, after a preliminary sudden drop, does diminish slowly for some days. Moloney and Weld (1922-5) have shown that the hydrogen-ion concentration of a toxin influences the rate of toxoiding. They show that between pH 7·0 and 10·0 the rate of decrease in toxicity is greater the more alkaline the solution. At pH 10·0, however, the solution loses its flocculating power. Moloney and Weld (1922-5) also show that whole cultures lose their toxicity in the presence of formaldehyde more readily than filtered toxin. These authors recommend as a routine procedure that formalin (3 c.cm. per litre) be added to whole cultures, which are then replaced in the incubator at 37° C. On the following day the pH is adjusted to 8·2 by the addition of sodium carbonate. After further incubation for four days, the material is ready for filtration. It must be pointed out, however, that the short exposure to a relatively low concentration of formaldehyde will not render all types of diphtheria toxin non-toxic. Ramon uses from 0·3 to 0·4 per cent. commercial formalin and leaves the culture for one month at 37° C. before filtration.

The rate at which a batch of toxin loses its toxicity may be shown by the following figures of Ramon (1924<sup>1</sup>). Formalin (0·35 per cent.) was added to a toxin and samples examined after incubation for various lengths of time.

Original toxin contained 800 m.l.d. per c.cm.					
After exposure for 1 day	50	"	"	"	"
"	"	3 days	10	"	"
"	"	"	10	"	"
"	"	"	20	"	"
"	"	"	30	"	"
				0	"

For general routine purposes the rate of toxoiding can be traced most conveniently by means of the intracutaneous test for m.r.d. In the majority of toxins which contain no other preservative, the flocculating value remains unchanged although the number of m.r.d. may fall from over one million per c.cm. to nil, showing that the total combining power remains unaltered while the specific toxin has become modified. If the term " toxoid " is used in a general sense for any modification of specific toxin which will still combine with antitoxin, but causes no symptoms in animals, it can be stated that toxin is not destroyed by formaldehyde

but is changed into toxoid. Unsatisfactory results are obtained if the original toxin contains phenol. Upon incubating in the presence of formaldehyde, precipitation and loss of antigenic value occur.

#### *Other Toxoiding Agents.*

Berthelot and Ramon (1925) have studied the action of a large number of substances upon toxin and have found that toxin is modified but not destroyed (i.e. the toxin loses its toxicity, but retains its flocculating and immunizing power) after 4 to 6 weeks exposure at a temperature of 38 to 40° C. by :

- 2·5 c.cm. of 7·4 per cent. acrolein per 100 c.cm. of toxin.
- 2·5 c.cm. of 9·3 per cent. crotonic aldehyde per 100 c.cm. of toxin.
- 4 to 5 c.cm. of 5·8 per cent. acetaldehyde per 100 c.cm. of toxin.
- 12 to 15 gms. of hexamethylene tetramine per litre of toxin.

Koulikoff and Kompanejez (1928) recommend the use of acetaldehyde since it is easy to remove by shaking or warming. Berthelot and Ramon (1925) consider acetaldehyde preferable to formaldehyde for the modification of tetanus toxin. Vallée and Bazy (1917) used Gram's iodine solution to modify tetanus toxin. In the author's experience, toxins modified by Vallée's method could be successfully used in the preliminary immunization of animals and are of particular use when supplies of already prepared formol toxoid or balanced toxin-antitoxin mixtures are not available. Nélis (1925) has shown that both ozone and salts of quinine have a considerable action on toxin, but his work does not indicate the extent to which modification, precipitation or destruction is brought about by these agents.

#### MODIFIED TOXIN, OTHER THAN TOXOID.

The change from toxin to toxoid must be regarded as an irreversible process. A definite alteration apparently occurs in the composition or structure of the toxin molecule, but toxin may be rendered temporarily harmless without such a definite change. Certain substances which delay absorption may, however, be added to a filtrate, so that it appears non-toxic when injected subcutaneously into animals. When these substances are removed, the filtrate regains its original toxicity. Such types of masked toxin differing fundamentally from toxoid have been aptly termed 'cryptotoxins'. Vincent (1907) found that the toxin could be masked but not destroyed by bile. A more important method of so masking a toxin appears to be that of Larson and his co-workers. Larson and Nelson (1924) showed that toxins treated with castor-oil soap were completely detoxified. Later, Larson, Evans and Nelson (1925) showed that 'soaps which detoxify bacterial toxins differ in physical properties from other soaps, in that they form clear, or nearly clear solutions in physiological sodium chloride solution, and dialyse readily through hardened collodion sacs. The detoxifying soaps depress the liquid-air

surface tension more than do other soaps of equivalent concentration'. They were led to 'the assumption that the detoxifying action of soap upon bacterial toxin is an adsorption phenomenon. The soap probably dissolves into the surface of the toxin molecule, thus imprisoning it so that it is not free to react with the tissues in the usual manner'. They further found that such detoxified toxins were good antigens. Larson, Hancock and Eder (1925) obtained a preparation innocuous to children and adults by mixing toxin with 1 per cent. solution of sodium ricinoleate and allowing the mixture to stand for six hours at room temperature before injection. They emphasize the importance of using only toxin-soap solutions which are perfectly clear and of keeping the soap solution as well as the soap-toxin mixtures in hard glass containers. Injections of cloudy solutions are invariably followed by severe local reactions. Larson, Halvorson, Evans and Green (1925) reported a series of experiments upon the effect of varying the proportions of toxin and sodium ricinoleate. To 0·4 c.cm. of toxin (1 L<sub>+</sub> dose) they added 1 c.cm. of 1, 2, 3 per cent., &c., sodium ricinoleate solutions; the first mixture killed a guinea-pig in 3 days, the second in 11 days and the third in 28 days. The mixture of toxin with 1 c.cm. of 4 per cent. solution of the soap did not kill a guinea-pig, but paralysis resulted. Higher concentration of sodium ricinoleate completely detoxified the toxin. A non-toxic mixture consisting of 0·4 c.cm. toxin with 1 c.cm. of 4 per cent. sodium ricinoleate, killed a guinea-pig in 3 days after the addition of 2·0 c.cm. of water. Mixtures of soap and toxin were also made toxic by precipitating the soap with calcium chloride. Nélis (1924) rendered toxin non-toxic by the addition of 4·0 c.cm. of 1 per cent. sodium oleate solution to 100 c.cm. of toxin, but the resultant product had little antigenic value.

Vincent (1928) has shown that there are other substances, e.g. sodium salicylate, sodium benzoate, &c., which modify or destroy tetanus toxin. He injected a guinea-pig of 450 gm. with 200 to 400 m.l.d. of tetanus toxin to which was added 1/30 saturated sodium salicylate solution and this injection caused no effect. Although the modified toxin has been shown to be antigenic, sufficient work has not been done to show whether much of the toxin has been destroyed and whether it still retains its combining power for antitoxin.

#### THE ACTION OF PHENOL ON TOXIN.

In all work on toxin, it is important to bear in mind the effects produced by the presence or absence of phenol. Any condition that tends to local concentration of phenol causes destruction, as in the shaking of dilutions. Moloney and Weld (1925) found that by using phenol as a preservative for toxin unsatisfactory results were obtained and in many cases the antigenic values dropped to zero. An example given by these workers may be quoted. The Lf of the original toxin used was 0·145; after 5 days at 37° C., the phenolized toxin had an Lf of 0·4, while the toxin alone had an Lf of 0·15. Glenny, Pope, Waddington and Wallace (1926<sup>a</sup>)

showed that the destruction caused by heating toxin containing 0·5 per cent. phenol to 45° C. is greater than that caused by heating the same toxin uncarbolized to 60° C. for the same length of time.

#### PRECIPITATION OF TOXINS BY SALTS.

The precipitation of toxin has important practical applications. Dried precipitated toxin forms a stable product often needed for test purposes as liquid toxin is frequently unstable, e.g. that of *B. tetani* and of *B. welchii*. For this purpose ammonium sulphate is a convenient precipitant. By various methods of precipitation a considerable degree of purification and concentration can be achieved, thus yielding material more suitable for chemical investigations and also enabling larger test doses to be used for titration, a procedure that should be especially useful with such weak toxins as those of dysentery, scarlet fever, &c. Toxin may be precipitated by many different salts, among which are ammonium sulphate, mercuric chloride, zinc chloride, copper acetate, uranium acetate and lead acetate, all of which have been used with varying degrees of success at different times. This work has been adequately summarized by Heinemann (1908) and by Pick (1912). No method has yet been successful in removing the non-specific material present in culture solutions which is so harmful in immunization. Probably this is bacterial protein, and it is always brought down with toxins on precipitation.

The sulphate method of precipitation is described by Heinemann (1908) who prepared concentrated toxin for horse immunization in the following way : 2 volumes of saturated ammonium sulphate are added to 1 volume of toxin with constant stirring. After standing 24 hours, the scum and sediment formed are removed, collected, dialysed for 5 days and then re-filtered. For most purposes, however, dialysis may be omitted. Rosenau and Anderson (1908) also use this method in preparing concentrated tetanus toxin. London and Aristovsky (1917) reported a new method of preparing a pure dry tetanus toxin. They added 17 per cent. of ammonium sulphate and discarded the centrifuged precipitate ; 1 to 3 per cent. of ammonium sulphate was then added to the liquid and the precipitate separated and dried *in vacuo* and then submitted to re-precipitation. This material will kill a white mouse in two days by intracutaneous injection of 0·00002 mgm., and there are indications that a further fractionation would result in a more toxic and purer material.

In 1926<sup>3</sup>, Glenny, Pope, Waddington and Wallace showed that toxoid precipitated by potash alum had a higher antigenic efficiency than the original toxoid. Further work on the increased antigenic efficiency of alum toxoid was carried out by Glenny and Waddington (1928). In the author's experience, tetanus toxoid to which 2 per cent. of alum has been added forms the most satisfactory agent for protecting horses used for the preparation of therapeutic sera. Wallace (1927) carried out further experiments on the use of alum as a precipitant and also used uranium

nitrate, Hedin's tannin solution, trichloracetic acid and ferric alum and aluminium nitrate. By the addition of these salts the zone of pH within which toxin can be precipitated is much wider than with acid alone. Hosoya and Miyata (1928) state that they have purified tetanus and diphtheria toxins by means of zinc chloride, and from the dialysate removed the zinc by precipitation. Abt (1928) has attempted to purify diphtheria toxin by adsorbing it on to calcium phosphate. After purification in this way, the yield was 57·9 per cent. of the original, the amount of protein decreased from 17·71 to 0·251 mgm. per c.cm., and the number of toxin units per mgm. of protein increased from 0·55 to 22·35.

#### PRECIPITATION OF TOXIN BY ACID.

It has long been known that toxin loses its toxicity upon the addition of acid and that a partial recovery of toxicity occurs upon neutralization. Glenny and Walpole (1915) showed that after preliminary dialysis through a collodion membrane both diphtheria and tetanus toxins were precipitated by the addition of a small quantity of acetic acid, and that most of the toxin as measured by combining power was recovered on dissolving the precipitate in dilute alkali. By this process, they obtained a yield of 70 to 80 per cent. of purified diphtheria toxin with a concentration of about 50-fold in terms of L<sub>+</sub> dose per mgm. of nitrogen. Toxin used by these authors was prepared from a beef peptone medium and was not precipitated by acid without preliminary dialysis. v. Gröer (1923) concentrated toxin by precipitation with hydrochloric acid. He found that the optimum pH for precipitation varied with the salt content and with the age of the toxins used.

Watson and Wallace (1924<sup>2</sup>) found that 1 per cent. glacial acetic acid precipitated toxin; Predtschensky and Scheviakovan (1926) showed that the maximum precipitate occurred when the hydrogen-ion concentration of the toxin was between pH 4·2 and 4·6 and the extreme limits of precipitation lay between pH 2·8 and 6·0. These authors used a number of different acids and found that the optimum pH for precipitation varied slightly with the acids used. A more complete study has been made by Wallace (1927) who found that two zones of precipitation occur at the acid end and one at the alkaline end. The limits of hydrogen-ion concentration at which precipitation occurred in the toxin examined by her were below 1, between 3 and 5 and over 9. The precipitate appearing between pH 3·0 and 5·0 was soluble upon adjustment, the alkaline precipitate was insoluble and the lower acid precipitate almost insoluble, but antigenic. Wallace (1927) found that at the point of optimum precipitation 60 per cent. of the antigen, estimated by flocculation, was precipitated and 40 per cent. remained in solution. With toxin containing 0·5 per cent. phenol the amount of toxin carried down in the precipitate between pH 3·0 and 5·0 is greater than that in toxin without phenol. In the batch of toxin tested, 14 per cent. only remained in solution at a pH of 4·4. Actual destruction is greater in the presence of phenol, and

the sum of toxin recovered from the filtrate and precipitate gradually falls with fall in pH. The extent of destruction increases with the length of time the toxin remains in the acid state. The amount of toxin that is precipitated varies considerably with the batch of toxin used. Sedallian and Gaumont (1927) found that toxin was precipitated at pH 4·7; they were not successful in recovering all toxin from the precipitate although the supernatant liquid failed to kill guinea-pigs in doses of 1·0 c.cm. The amount of toxin remaining was measured in terms of fatal doses; the apparent loss may have been due to modification and not to destruction. These workers found that no acid precipitate formed in old toxin nor in toxin modified by formaldehyde. Koulikoff and Smirnoff (1927) found that the hydrogen-ion concentration at which the maximum active principle was precipitated from a certain toxin did not correspond with that at which the maximum precipitate was found. At pH 4·8, over 90 per cent. of toxin was recoverable from the precipitate, but the actual precipitate formed was only 40 per cent. in weight of that produced at pH 3·8, from which only a fraction of the original toxicity was recoverable.

With old or formolized toxin, the point at which the optimum yield of antigen is obtained coincides with the point of maximum precipitation. Koulikoff and Smirnoff (1927) suggest that other batches of toxin may yield very little precipitate at the true isoelectric point of the specific antigen; such toxin would give the purest preparation obtainable by acid precipitation.

The extent to which modification occurs upon the addition of acid or alkali has not been fully traced. There is considerable evidence to show that some of the toxin is changed into toxoid upon the addition of acid. The early observation that acid destroyed toxicity but upon neutralization with alkali part of the toxicity returned, can be explained by the occurrence of partial modification and precipitation on the addition of acid. On neutralization, both toxin and toxoid returned to solution, but some of the toxin had been modified into toxoid. An alternative improbable explanation that has been suggested is that the destructive action of the acid solution upon the tissues of the guinea-pigs injected delayed absorption of the toxin.

Moloney and Weld (1922-5<sup>2</sup>) and Bächer, Kraus and Löwenstein (1926) have further purified toxin and toxoid by alcoholic precipitation of the dissolved acid precipitate.

Although acid precipitation of toxin effects a considerable concentration (50- to 200-fold) of antigen in terms of nitrogenous matter, it appears that bacterial protein is also precipitated, and in the author's experience such concentrated toxins do not form suitable material for the immunization of horses. Glenny and Waddington (1928) show that the antigenic value per Lf unit of acid-concentrated toxoid is less than that of the crude toxoid and they suggest that non-specific material harmful to antigenic response is all precipitated by acid; only about 50 per cent. of specific toxoid is

precipitated. It follows, therefore, that the concentration of harmful non-specific material in comparison with necessary specific antigen is increased by this method of concentration.

#### THE ACTION OF HEAT ON TOXIN.

Dean (1908) states that 'exposure to a temperature of 58° C. for 2 hours or 100° C. for 20 minutes almost completely destroys the toxicity of filtrates. Toxin precipitated with calcium phosphate and dried is much more resistant; it withstands a temperature of 70° C., and it is not much affected even by a temperature of 100° C. for 20 minutes'. De Potter (1924) has shown that toxin heated for 18 hours at 50 to 55° C. produces no appreciable immunity, but slight immunity may be produced by the injection of toxin heated for 20 minutes at 80° C. Glenny, Pope, Waddington and Wallace (1926) found that unpreserved toxin lost 10 per cent. of its combining power after an hour at 45° C., 35 per cent. at 50° C., 70 per cent. at 55° C. and about 80 per cent. at 60° C. Carbolized toxin lost over 90 per cent. after 1 hour at 45° C., 30 minutes at 50° C. or 15 minutes at 55° C. These figures refer to total content of toxin and toxoid measured by the flocculation test, while Dean refers to destruction of 'toxicity' which may be brought about partly by total destruction and partly by modification. The extent to which toxin is modified by heat does not appear to have been investigated.

Toxoid produced by the action of formaldehyde on toxin is much more stable than toxin. Glenny, Hopkins and Pope (1924) showed that 'modified toxin' and concentrated modified toxin can be boiled without destroying entirely their antigenic properties. Glenny, Pope, Waddington and Wallace (1925<sup>3</sup>) showed that some antigenic power remained after boiling modified toxin for five minutes. In a precipitated form, not necessarily dried, toxoid is very stable. Glenny, Pope, Waddington and Wallace (1926) state that 'a suspension of alum precipitate after boiling for one hour was equal in antigenic value to the average toxin-antitoxin mixture used for human immunization'. Ramon (1924<sup>2</sup>) showed that 'anatoxine', the term used by him to describe modified toxin, could be heated to 65° C. for 1 hour without any reduction in combining power. No experiments appear to have been made upon the heat-stability of toxoid produced by any method other than by the action of formaldehyde. It is not possible, therefore, to state that every type of toxoid is heat-stable or to decide what effect the alteration in the medium produced by the action of formaldehyde has upon the heat-stability. Ramon (1924<sup>2</sup>) showed that the increase of resistance to heat during modification did not occur equally with the decrease in toxicity. After one day's incubation in the presence of formaldehyde, the toxicity had fallen from 800 m.l.d. per c.cm. to 50, but no flocculation reaction could be obtained after heating to 65° C. for 1 hour. By the tenth day, the toxicity had fallen to 1 m.l.d. per c.cm., and the product was more stable to heat, but the flocculation was slow. Although less than 1 per cent. of toxin remained,

about 50 per cent. of the combining power was destroyed upon heating, and yet when, at the end of 30 days, all toxicity was lost, there was no loss in combining power upon heating to 65° C. for 1 hour. These results cannot be explained by a difference in stability of toxin and toxoid unless the final product reached when all toxicity has gone is different from the toxoid during the course of modification. It is probable that the alteration of the surrounding medium has some effect upon heat-stability. Koulikoff and Smirnoff (1927) state that toxin can be stabilized to heat by the addition of a ' fermentoid '.

#### FREEZING OF TOXIN.

Freezing of toxin containing phenol causes concentration of phenol and destruction. For example, toxin stored in the cold room retains its toxicity, but when actually frozen, there is a marked fall in toxicity. In the absence of phenol such loss does not occur. Banzhaf (1928) states that toxin is destroyed by saturation with sodium chloride and that freezing destroys about half the toxin. This does not agree with the author's experience with toxin containing no preservative.

#### STABILITY OF TOXIN.

The change that takes place in diphtheria toxin kept for a considerable period at room temperature is chiefly a slow modification of toxin to toxoid ; this is an advantage rather than a disadvantage if the toxin is for use in immunization. It is not known at present, however, whether old toxins undergo any change effecting an alteration of avidity. Toxin intended for test purposes usually contains 0·5 per cent. phenol and is preserved under a layer of toluene in amber-coloured hard glass bottles at a temperature just above freezing-point. After a lapse of from six months or a year after preparation, toxin may remain stable for many years. Glenny and Südmersen (1921) refer to a toxin in constant use with an L+ dose remaining unchanged for 13 years. Usually, however, the L+ dose gradually increases although the L<sub>0</sub> may remain unaltered. In the author's experience, rapid changes occur in test toxins in constant use when only a small volume of toxin remains in a bottle. There is no reasonable explanation of this, but it is of great practical importance. MacConkey (1923) found that the addition of 50 per cent. glycerin stabilized liquid tetanus toxin. Banzhaf (1928) adds three parts of glycerin to one of diphtheria toxin for use in the Schick test.

Toxoid is very stable. The author has rendered guinea-pigs Schick-negative by a single injection of formolized toxin kept at room temperature for 24 years. Dilutions of toxoid are less stable.

The stability of dilutions of toxin is of practical importance in the preparation of material for the Schick test. Glenny, Pope and Waddington (1928) suggest the use of a borate buffer solution as diluent in place of phenol saline. These authors correlate the stability of the toxin dilution at room temperature with the rate of destruction after 24 hours' exposure

at a temperature of 35° C. Glenny and Waddington (unpublished) find that the addition of broth to the toxin dilutions further increases the stability, so that diluted Schick toxin may be kept at room temperature for six months without appreciable loss. Leulier, Sé dallian and Clavel (1928) found that toxin or toxoid precipitated at pH 4·7 is unstable unless resolution is made in an alkaline solution of peptone or broth. They suggest that proteoses give colloidal protection.

Scarlet-fever toxin is more heat-stable than diphtheria toxin, while other toxins such as those of the pathogenic anaerobes are less stable. The stability of individual batches of both tetanus and *B. welchii* toxins have, in the experience of the author, varied considerably. Thus, of the toxins prepared and used for horse immunization during the war, tetanus toxin rapidly lost its toxicity while retaining its combining power and *B. welchii* toxin appeared stable in the liquid state for many months. In recently prepared toxins, the reverse has been the case. It is known also that diphtheria toxoid produced by the action of formaldehyde is far more stable than toxin. It is possible that the stability of all toxins depends on some physical or chemical property of the surrounding medium or of the supporting colloid. It is interesting to note that Willstätter (1927) states that enzymes must be regarded as being composed of a specifically active group and a colloidal carrier, which may vary in its nature, but is necessary for the stability of the active group.

#### SHAKING OF TOXINS.

Glenny, Pope, Waddington and Wallace (1925<sup>3</sup>) studied the effects of shaking dilutions of diphtheria toxin of Schick strength in 0·5 per cent. phenol saline, and in many cases found that the toxin was almost completely destroyed. After various experiments it was ascertained that in cases where the bottles containing the dilutions were quite full no loss occurred, but marked destruction resulted in bottles which were only half full, and this destruction increased with relative decrease in volume of the dilution. This is due to the concentration of toxin and phenol at the air-liquid surface. Undiluted toxin was far less susceptible to destruction by shaking. Pope (1927) has demonstrated the degree of concentration of phenol that may occur at the surface when solutions are shaken.

#### Action of Chemical and Physical Agents on Antitoxin.

Work in this field has been generally limited to experiments upon the stability of antitoxic sera stored under different conditions and to methods of purifying antitoxin. It may be stated generally that antitoxin is so closely linked to pseudo-globulin that the physical and chemical properties of antitoxin are the same as those of pseudo-globulin. Any process such as dialysis or salt precipitation that removes euglobulin or albumin from serum leaves antitoxin intact, and conversely, any method that precipitates the pseudo-globulin brings down the antitoxin also. There are, however,

suggestions that antitoxin may be freed from the bulk of the pseudo-globulin. The precipitates which form when toxin unites with antitoxic serum weigh considerably less than the pseudo-globulin of the added serum.

By the action of acid upon toxin-antitoxin floccules, Ramon (1923<sup>1</sup>) produced diphtheria antitoxin in a purer form than has yet been produced by any method of salt precipitation of pseudo-globulin. Both he, and Locke and Main (1926) quote 60,000 units per gm. Banzhaf (1928) was able to show that on heating the serum or plasma about 30 per cent. of the pseudo-globulin is converted into a substance resembling euglobulin. On heating for long periods there was no further conversion and much antitoxin was lost. Heating in the presence of 30 per cent. saturated ammonium sulphate converted a further 10 per cent. of the pseudo-globulin.

#### *Stability of Antitoxin.*

The influence of age and temperature on the potency of antitoxin has considerable practical importance. Anderson (1910) gives the average yearly loss as 6 per cent. at 5° C., 10 per cent. at 15° C., and 20 per cent. at room temperature varying between 70° F. in winter to 95 or 100° F. in summer. MacConkey (1912) found that at 36° C. the rate of loss was 36 per cent. in 6 months and 50 per cent. in a year. The following figures are typical of many results upon the loss of antitoxin in sera containing 0.3 per cent. tricresol left for 12 months at various temperatures : 0 per cent. at 0 to 5° C., 9 per cent. at 15° C., 11 per cent. at 22° C., 20 per cent. at 30° C., 60 per cent. at 37° C. In the absence of antiseptic, the rate of loss is far less and may be as little as 20 per cent. in 12 months at 37° C.

From the practical standpoint, it is important also to know the rate of loss of 10 per cent. dilutions of antitoxin such as are used in the Schultz-Charlton reaction. There is no reason to suppose that different antitoxins vary in their stability, the figures for diphtheria antitoxin can therefore be quoted as probably applicable to scarlet-fever antitoxin. The average of several sera diluted 1 in 10 in phenol saline showed a loss in 12 months of 28 per cent. at 15° C. and 63 per cent. at 37° C.

Destruction appears to depend upon the coagulation of the protein. In dilution, antitoxin is more heat-stable ; thus the author has found a 1 in 50 dilution of serum in phenol saline to contain more antitoxin after boiling for 1 hour at 100° C. than a 1 in 10 dilution after the same treatment. Phenol appears to have some protecting action because no antitoxin was demonstrated after boiling dilutions in saline without phenol. It is probable that the amount of destruction depends upon the pH of the diluting fluid.

MacConkey (1923) states that antitoxic serum saturated with common salt remains sufficiently stable to justify its use as a test serum. The addition of 50 per cent. glycerol helps to stabilize antitoxin. Standard

antitoxin preserved at the three Standards Laboratories is prepared by drying antitoxic serum containing no antiseptic and keeping as a dry powder *in vacuo*. Periodically known amounts of the dried antitoxin are dissolved in 50 per cent. glycerin solution and issued to serum manufacturers to enable them to check their test toxin.

### The Action of Chemical and Physical Agents upon Toxin-Antitoxin Mixtures.

Toxin-antitoxin mixtures are still used in a number of countries for the active immunization of children against diphtheria. It is of importance, therefore, to consider the stability of such mixtures and the conditions under which they may become toxic. Kelley (1924) reported that toxin-antitoxin mixtures which had become frozen had caused severe reactions on injection. This phenomenon was investigated at once by a number of workers with varying results. White and Robinson (1924) and Anderson and Leonard (1924) succeeded, while Kirkbride and Dow (1924) failed to bring about such increased toxicity on freezing. The work of Anderson and Leonard (1924) indicated that such increase in toxicity only occurred in the presence of phenol or tricresol. Glenny, Pope, Waddington and Wallace (1925<sup>3</sup>) showed that certain strengths of phenol around 5 per cent. caused a greater relative destruction of dilutions of antitoxin than of toxin and that the addition of this amount of phenol to a non-toxic mixture may render it toxic. They suggested that the local concentration of phenol which occurs when the mixture is frozen causes a similar effect. Pope (1927) demonstrated that there was separation of phenol upon freezing, and that the extent of separation depends upon the protein and salt-content and upon the temperature and duration of freezing. These variables would account for the discrepant results obtained by different workers. Banzhaf and Greenwald (1924) found that toxin-antitoxin mixtures became toxic by freezing for 18 hours at  $-8^{\circ}\text{C}$ . This toxicity increased with time and temperature of freezing. Three weeks after thawing the mixtures were found to be non-toxic. Robinson and White (1928) also showed that mixtures that had become toxic on freezing lost this toxicity after thawing, but became toxic again on re-freezing. From this work it would appear that antitoxin is not fully destroyed by the phenol but is rendered relatively insoluble. Glenny, Pope and Waddington (1925<sup>1</sup>) showed that mixtures of toxin and certain modified antitoxin apparently increased in toxicity upon dilution with saline solutions.

Ramon (1923<sup>2</sup>) showed that salt-free toxin-antitoxin floccules dissociate upon the addition of 1 in 1,000 to 1 in 1,500 acetic acid. Upon heating for 1 hour at  $58^{\circ}\text{C}$ . toxin is destroyed and antitoxin remains. Glenny and Pope (1927) found that the optimum recovery of antitoxin from toxoid-antitoxin floccules occurred at pH 3.5. Madsen and Schmidt (1926) state that both toxin and antitoxin can be recovered from floccules by the action of sodium iodide.

### Combination Between Toxin and Antitoxin.

From time to time, various theories have been put forward to account for the phenomena concerning the nature of diphtheria toxin and antitoxin, and the combination between them. These phenomena are as follows :

1. The number of fatal doses of toxin which can be neutralized by a given amount of antitoxin varies with the batch of toxin used.
2. As a toxin ages, the minimal amount required to kill a guinea-pig gradually increases, but the quantity which can be neutralized by a given amount of antitoxin remains comparatively constant.
3. Toxin may be so modified that it will no longer kill a guinea-pig but will still combine with the original amount of antitoxin.
4. Successive equal increments of antitoxin added to the same toxin do not reduce toxicity equally.
5. The differences between the L+ dose of a toxin and the Lo dose is greater than 1 m.l.d. (Ehrlich phenomenon).
6. A given amount of antitoxin neutralizes less toxin when the toxin is added in stages than when all the toxin is added at once (Danysz effect).
7. An Lo mixture of toxin and antitoxin may produce late paralysis in guinea pigs while sublethal doses of toxin alone rarely produce paralysis.
8. Neutral (Lo or Lf) mixtures of toxin and antitoxin are antigenic.
9. Both toxin and antitoxin can be recovered from neutral mixtures by chemical and physical means.
10. A modified form of antitoxin can be produced, which, when mixed with toxin and injected into guinea-pigs, will prevent the formation of a local swelling, but will not prevent subsequent death of the animal.
11. Mixtures of such modified antitoxin with toxin may produce no reaction when injected undiluted, intracutaneously, into guinea-pigs, but reactions may occur when a high dilution of the mixture is injected.
12. The antitoxic titre of a serum determined by the in-vitro method may vary from  $\frac{1}{2}$  to 3 times that obtained by the in-vivo method, although in the large majority of sera tested, there is close agreement between values obtained by the two methods.

### MODIFICATION OF TOXIN.

It is evident that the existence of at least one modification of toxin must be assumed.

#### *Toxoid.*

Ehrlich originally introduced the term ' toxoid ' for that modification of toxin which would no longer produce symptoms when injected into guinea-pigs, but was still capable of combining with antitoxin. The assumption of the existence of this modification was based primarily upon the fact that a given amount of antitoxin neutralized varying numbers of m.l.d. according to the toxins used. The assumption was further justified when it was found that with a given test toxin the Lo dose remained constant for some time while the toxicity gradually fell.

Ehrlich's original simple conception of toxin and toxoid became unnecessarily complicated by his assumption that there were many distinct forms of toxoid differing from one another in their affinity for antitoxin. These complications generally resulted from the conception that toxin and antitoxin became firmly united, combining in the same way as strong acids with strong bases. Recent work, however, supports the idea of loose combination after the manner of the weak acid-base combination, first suggested by Arrhenius and Madsen (1902). This theory does not need the assumption that there exist many different forms of toxoid.

It has long been known that toxoid as well as toxin is antigenic; thus, Glenny and Walpole (1915) state that a batch of diphtheria toxin is estimated by the immunologist according to its 'binding unit content' per c.cm. Glenny and Südmersen (1921) first showed that diphtheria toxin could be entirely changed to toxoid while preserving its antigenic properties. These workers modified toxin by the action of formaldehyde; 5.0 c.cm. of the material no longer killed a guinea-pig but was still antigenic.

Ramon (1923<sup>8</sup>) introduced the term 'anatoxine' for formolized toxin rendered completely atoxic. The suggestion that 'anatoxine' is not synonymous with toxoid does not appear justified for the following reasons. Ramon suggests that three properties distinguish 'anatoxine' from toxoid: its flocculating power, its non-toxicity, and its stability. The flocculating values of various diphtheria filtrates harvested after different times of growth, and, therefore, containing different ratios of toxoid to toxin, do not bear a constant ratio to the m.l.d. It follows, therefore, that the relation between m.l.d. and Lf is similar to the relation between m.l.d. and Lo, which led Ehrlich to deduce the presence of toxoid. Toxoid, therefore, has a flocculating value. The relative stability of the Lo dose of toxin compared with the instability of the m.l.d. indicates that toxoid is more stable than toxin. Recent work, mainly by Koulikoff and his co-workers, has shown that the stability of toxin or antitoxin may be greatly affected by the physical and chemical properties of the surrounding medium. Hence the remarkable stability of formolized toxin may not be entirely a function of the specific toxoid or 'anatoxine'. Ehrlich's original definition of toxoid included a statement that it was harmless; thus it is difficult to see that there is any justification in regarding toxin formolized by Ramon as differing essentially from such toxoid as is present in all filtrates.

It is not yet known whether toxoid produced in different ways varies in chemical constitution. There may be many forms of toxoid differing slightly according to the method of preparation, for example, the action of formaldehyde, heat, or unknown influences which may be exerted when toxins are preserved for a considerable period. There are two obvious conceptions of toxoid. First, that the toxic radical has combined or has been removed; and secondly, that some chemical combination with toxin

or an aggregation of toxin molecules so increases the size that they do not rapidly diffuse into living cells, and are not absorbed until broken down in some manner which renders them inert, or that they are broken down so slowly that the concentration of toxin never reaches a lethal value. Schmidt and Scholz (1925) suggested that toxin and toxoid differ only in their degrees of dispersion and brought forward to support it confirmation of the experimental work of Walbum (1909) that fresh toxin increases considerably in toxicity after short incubation in the presence of broth. Schmidt and Scholz (1926) found that after a toxin was diluted fourfold in broth and incubated, the m.l.d. increased by barely 50 per cent. although the volumes of the L+, Lo, Lf increased fourfold. The dilution with broth has increased the amount of toxin present presumably at the expense of pre-existing toxoid. The number of m.l.d. per Lf was 80 in the original toxin, 170 after incubation with an equal volume of broth, 210 with three times its volume and 200 with seven times the volume of broth. It would appear that however loose the aggregation may be in a fresh toxin, a stable condition is soon reached and toxoid can no longer be changed to toxin. These experiments, as yet unconfirmed, suggest that toxin and toxoid may be regarded as the same substance in different stages of dispersion. Two possibilities then present themselves: (1) as a toxin filtrate ages some of the toxin remains unchanged and some aggregates to form toxoid, or (2) all the toxin suffers a gradual change and the extent of dispersion decreases throughout. The latter appears the more reasonable hypothesis, but until more data are available it appears preferable to deal with the many problems that arise on the assumption that a culture filtrate consists of varying proportions of two substances, toxin and toxoid, rather than one substance in varying stages of modification. The experimental data of Schmidt and Scholz (1925) call for one comment: upon apparent dispersion the L+ dose does not approach nearer the Lo or Lf, as would be expected with increased toxicity. Thus, these results do not appear to be a simple reversal of the ageing of toxin, when the L+ and Lo differ more widely as the toxicity decreases.

#### *Toxone.*

Ehrlich, observing that the L+ and Lo dose of a diphtheria filtrate differed by more than 1 m.l.d., assumed the presence of a toxoid with less affinity for antitoxin than toxin and he called the modification 'epitoxoid'. The later observation that paralysis was caused by Lo mixtures, i.e. those in which the toxin was completely neutralized by antitoxin, induced Ehrlich to rename the material 'toxone', which he considered was a primary secretion of *C. diphtheriae*, differing from toxin in that it caused late paralysis and not acute death and had less affinity for antitoxin. Dreyer and Madsen (1902) suggested that paralysis was due to dissociation of the toxin-antitoxin complex. There appears to be no necessity for the assumption of a separate substance, toxone.

## PARTIAL SATURATION OF TOXIN BY ANTITOXIN.

Ehrlich (1898) studied the relative affinities of toxin and toxoid for antitoxin by a partial neutralization method. Various fractions of a unit of antitoxin were added to one Lo dose of toxin and the mixtures titrated for residual toxicity. It was found that successive equal increments of antitoxin added to the same toxin did not reduce the toxicity equally. The addition of the first quantity of antitoxin did not seem to diminish the toxicity of a number of toxins examined. Ehrlich therefore concluded that some of the toxoid could bind antitoxin before the toxin itself, and called this theoretical material 'proto-toxoid'. Subsequent additions of antitoxin greatly reduced the toxicity, but the amount of toxin bound became gradually less at each addition until much antitoxin was needed to bind the last traces of toxin.

The experimental facts in support of the failure of the first addition of antitoxin to bind toxin do not appear satisfactory. Without a large number of guinea-pigs, it is not possible to determine differences of 10 per cent. in toxicity, and the dilution of a toxin-antitoxin mixture necessary for the determination of the number of m.l.d. present may cause some dissociation between antitoxin and toxin, so that there is less apparent reduction in toxicity upon the addition of the first amount of antitoxin. Ehrlich believed that further neutralization took place in abrupt stages, but such belief is not supported by later work. Arrhenius and Madsen (1902), working with tetanolysin, showed that the curve of residual toxicity upon successive additions of antitoxin was a continuous one. Similar smooth curves have been published by Henry (1922) for *B. welchii* haemolysin, by Glenny, Pope and Waddington (1925) for diphtheria toxin and by Buttle and Trevan (1928) for the action of *V. septique* toxin on strips of isolated uterus.

Various attempts have been made to establish analogies with the partial saturation curve of toxin and antitoxin. Ehrlich assumed that combination was complete—that all the antitoxin added combined with antigen. Arrhenius and Madsen assumed that antigen and antitoxin combine to form a reversible complex, the reaction being governed by the law of mass action and being of such a nature that appreciable amounts of both free antitoxin and toxin exist simultaneously, together with the toxin-antitoxin complex. Bordet (1903) and Craw (1905) considered that the combination was of the nature of an adsorption compound such as is formed when a dye combines with paper. The evidence for these different analogies is extensive and inconclusive, the fitting of curves with three or four arbitrary constants is a misleading procedure and many alternative theories can be developed in this manner from the same body of data. It would take more space than is available to discuss all these theories thoroughly, but in the following paragraphs we have tried to outline, with the minimum number of assumptions, what may be the kinetics of the reaction.

Four possibilities are shown in Chart II, Figs. 1 to 4: 'Total antigen (toxin and/or toxoid)' is shown on the ordinates and 'Amount of antitoxin' is shown on the abscissæ. The upper curve in each figure represents the total antigen remaining after the addition of different amounts of antitoxin, the lower curve the amount of toxin free; the difference between the curves is the amount of toxoid remaining. The total antigen remaining unneutralized is represented as lying along a straight line. This involves the assumption that the affinities of toxin

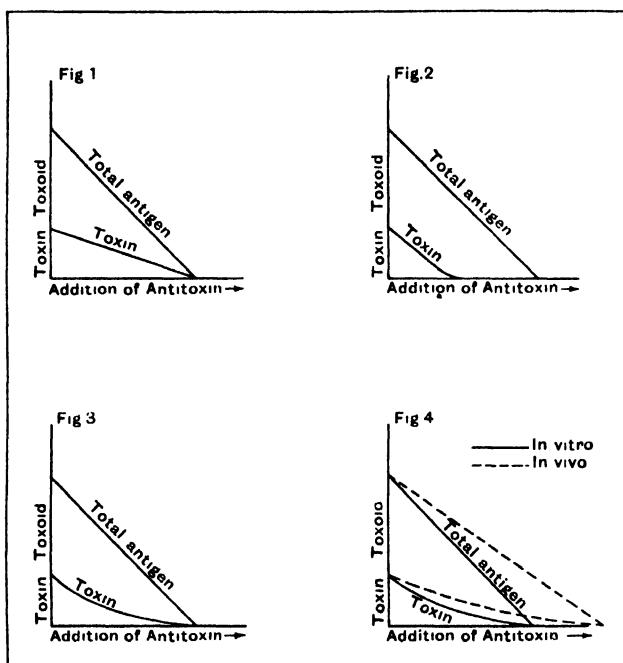


CHART II. Diagrammatic representation of partial saturation of toxin assuming that equal increments of antitoxin combine with equal amounts of total antigen (toxin + toxoid). Fig. 1: if toxin and toxoid have equal affinity for antitoxin. Fig. 2: if toxin has a much greater affinity than toxoid. Fig. 3: if toxin has a greater affinity than toxoid. Fig. 4: if toxin has a greater affinity than toxoid and combination is so loose that some dissociation occurs when mixtures are injected into animals.

and toxoid for antitoxin are so great that no appreciable amount of antitoxin is free in the mixture at any stage until all the toxin is combined with antitoxin. If antitoxin is present in any considerable amount before excess is added the upper line would be convex toward the abscissa, but the argument as far as it is developed will be unaffected.

#### RELATIVE AFFINITY OF TOXIN AND TOXOID FOR ANTITOXIN.

If it is assumed that equal increments of antitoxin combine with equal amounts of total antigen, i.e. toxin plus toxoid, then, if toxin and toxoid have equal affinities for antitoxin, equal proportions of toxin and

toxoid would be neutralized at each successive stage of partial neutralization as shown in Fig. 1. If, however, toxin has a much greater affinity than toxoid almost all toxin would combine with antitoxin before any toxoid enters into combination, as shown in Fig. 2. If the affinity of toxin for antitoxin is not very much greater than that of toxoid, the first addition of antitoxin will neutralize relatively more toxin than toxoid, but the ratio of the amounts neutralized would gradually lessen as the proportion of free toxin to toxoid decreased. Such conditions are shown in Fig. 3.

Figs. 1 to 3 show hypothetical conditions existing *in vitro*. If the combination between antitoxin and antigen is such that a splitting up of the combination can occur *in vivo*, some free toxin may combine with the tissues and thus determinations *in vivo* would differ from those *in vitro*. Such conditions are depicted in Fig. 4.

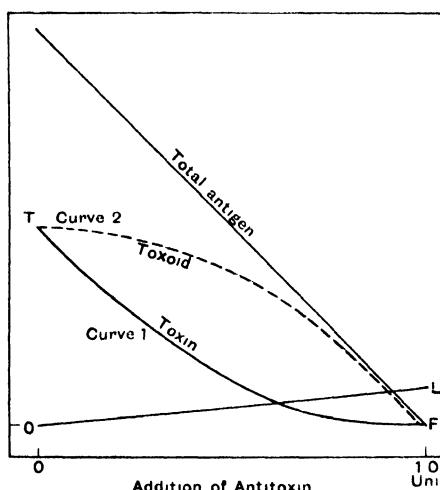


CHART III. Curves of partial saturation with antitoxin of toxin, toxoid and total antigen.

Curve 1 on Chart III has been drawn from experimental data published by Glenny, Pope and Waddington (1925) and corresponds generally with the diagrammatic representation in Chart II, Fig. 3. The curve shows the relation between the amount of toxin uncombined and the amount of antitoxin added to a given volume of partially modified toxin. Sufficient detailed experimental work has not been done to establish the relative shapes of the curves for different toxic filtrates varying in their toxin-toxoid content; an exact formula, therefore, cannot yet be assigned to the reaction. For diagrammatic purposes, we have accepted Curve 1 in the following discussion as representing the general characteristics of the curve of neutralization of any toxin. If all the antitoxin added enters into combination then the neutralization of the total antigen is represented by a straight line, and the amount of free toxoid can be plotted from the differences between total antigen and toxin. The hypothetical toxin depicted on Chart III is assumed to contain equal parts of toxin and toxoid.

*Relation between Units of Combining Power.*

It is generally assumed that in an Lf mixture of toxin and antitoxin there are present equal parts of antigen and antibody, i.e. that a mixture of equivalent parts of antigen and antibody flocculates more rapidly under the same conditions than any mixture containing other proportions. It follows, therefore, that if OF in Chart III represents one unit of antitoxin OT represents the amount of toxin in one Lf dose.

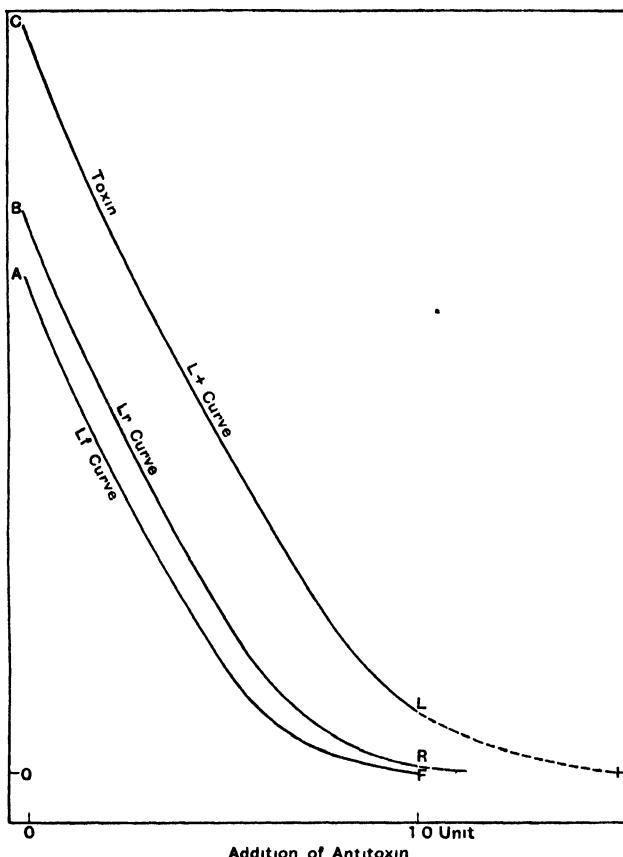


CHART IV. Curves showing partial saturation with antitoxin of the specific toxin in an Lf, Lr and L+ dose of toxin and of toxoid in the L+ dose.

At some point slightly to the left of F there is sufficient toxin free to cause a definite skin reaction if the mixture be injected intracutaneously into guinea-pigs. At a point still further to the left there is sufficient free toxin to kill a guinea-pig. These points do not correspond to the Lr and L+ doses because they represent the partial neutralization of a fixed dose of toxin (one Lf) by less than one unit of antitoxin, rather than of a varying dose of toxin by a fixed amount of antitoxin (one unit). If, however, from a point L above F representing one m.l.d. a line be drawn

to the origin O, the abscissa of the point where this line intersects the Lf curve bears the same relation to OF as the Lf dose to the L+ dose.

Chart IV shows three parallel curves : the Lf curve shows the complete course of neutralization of an Lf dose of toxin by one unit of antitoxin, the other curves are drawn through points R and L above F. If the ordinate FL represents the amount of free toxin that will kill a guinea-pig on the fourth day, then the curve passing through L is the neutralization curve for an L+ dose of toxin, i.e. that amount of toxin which when mixed with one unit of antitoxin will kill a guinea-pig on the fourth day. Similarly, if FR is equal to the smallest amount of toxin detectable by animal tests, i.e. 1 m.r.d., the curve passing through R represents the curve of neutralization of an Lr dose of toxin. The ordinates OA, OB and OC represent the amount of specific toxin in the Lf, Lr and L+ doses respectively and are therefore proportional to the volumes of these doses. The Lr dose has been depicted on the chart because it presents a more definite end point than the Lo dose ; the former depends upon the presence of a small definite excess of toxin and the latter upon the absence of a less easily determined but slightly larger quantity of free toxin. As a toxin ages or is artificially modified some toxin changes into toxoid ; the combining power remains constant, but the number of fatal doses per unit of combining power (Lf) decreases. Consideration of Chart IV shows that the L+ dose must increase. The distance OA represents the amount of specific toxin neutralized by the antitoxin OF (1 unit). If the number of m.l.d. per Lf falls, the point A is lowered, but the point F is fixed. The curve therefore becomes less steep in every section, the proportional curve drawn through L must also be less steep and therefore reaches the base line to the right of H. As OA decreases, OH increases, but OF is constant ; therefore, as A falls the ratio of L+ to Lf increases. It can be shown from the relative curvatures at L and R that the ratio of Lr to Lf does not increase so rapidly as the ratio L+ to Lf with a decrease in m.l.d. per Lf dose. This is more evident when it is realized that on Chart IV the distance FR has been greatly exaggerated in comparison with FL ; FR representing 1 m.r.d. is less than one-thousandth of FL, representing 1 m.l.d. These facts agree with the observation that as the toxicity of a toxin falls, the L+ dose increases more rapidly than the Lo dose. Typical figures for a series of toxins of different ages are given in Table III compiled from results published by Schmidt and Scholz (1925), and, from a series of successive modifications of one batch of toxin, in Table IV, from results obtained by Glenny, Pope and Waddington (1925).

Apart from differences in toxin and toxoid content of a filtrate there are definite differences in quality of antitoxin.

Many authors agree that the mixture of toxin and antitoxin in which specific flocculation first takes place, is not necessarily neutral to animals. Thus, Glenny and Pope (1927) tested Ramon's 'précipite indicateur' formed by mixing a given toxin with 3 different antitoxins and found that in 1 c.cm. the 3 mixtures had an excess of 18 units of antitoxin,

0·1 unit of antitoxin and 5,000 m.r.d. of toxin respectively. Other authors have called attention to frequent discrepancies between *in-vivo* and *in-vitro* values of different sera ; this figure varies from 1 : 3 to 3 : 1.

Such differences may be explained by reference to Chart II, Figs. 2 and 4. The majority of samples of antitoxic sera unite with toxin in

TABLE III.

Showing the dependence of the ratio of measurements of combining power upon specific toxicity of toxins of different ages.

Date of Preparation.	m.l.d. per Lf.	Lo/Lf.	L+/Lf.
January, 1925 .. .. .. ..	132	1·00	1·48
February, 1925 .. .. .. ..	88	1·06	1·25
January, 1925 .. .. .. ..	71	1·05	1·41
December, 1924 .. .. .. ..	66	1·04	1·51
August, 1924 .. .. .. ..	56	1·06	1·92
December, 1921 .. .. .. ..	36	1·34	2·19
August, 1921 .. .. .. ..	26	2·07	2·76
August, 1923 .. .. .. ..	22	2·25	2·66
1904 .. .. .. ..	4	2·15	3·96

TABLE IV.

Showing the dependence of the ratio of measurements of combining power upon specific toxicity of a toxin during successive stages of modification.

m.l.d. per Lf.	Lo/Lf.	L+/Lf.
55	1·64	1·91
37	1·73	2·00
11	1·82	2·00
5	2·09	2·54
2	2·66	3·17

such firm combination that the curve of neutralization of toxin by such antitoxin is that depicted in Chart III and Chart II, Fig. 3. The Lf point represents a complete balance between antigen and antibody ; the Lo and Lr doses will be only slightly greater than the Lf dose because these doses mixed with one unit of antitoxin have only a minute amount of toxin free. If, however, the combination between antigen and some specimens of antibody is so loose that dissociation occurs when mixtures are injected into animals, then the Lf mixture containing equivalent parts of antigen and antibody will apparently contain sufficient toxin uncombined

to cause reaction or death in the injected animals ; thus, more antitoxin is needed for in-vivo neutralization, as shown in Chart II, Fig. 4. This phenomenon suggests that the complete explanation of the toxin-antitoxin reaction must include consideration of the physical state of the antitoxin and therefore the reaction cannot be explained on simple chemical lines.

Investigation of antitoxic sera where the in-vitro value is greater than the in-vivo value has shown that they correspond to the modified antitoxin described by Glenny (1913). Glenny, Pope and Waddington (1925) have shown that such mixtures are slow to flocculate and are easily dissociated, dilution alone being sufficient to produce an apparent increase in toxicity. Thus discrepant ratios are easily explainable.

On the other hand, if other samples of antitoxin do not dissociate so easily as average samples the greater affinity of toxin over toxoid for antitoxin will be more apparent and the curve of neutralization follows that depicted in Chart II, Fig. 2 ; considerably less antitoxin is needed to reduce the amount of free toxin to less than that which causes reaction in animals than is needed for the complete balance necessary for flocculation. These differences between in-vitro and in-vivo titrations of antitoxin suggest the need of an official standard flocculating serum.

#### ACCURACY OF TESTING FOR M.L.D. AND L+.

Graphical explanations may be used to demonstrate the relative degrees of accuracy of testing for m.l.d. and for L+. Thus in Chart V, Curve 1 represents the partial neutralization of an Lf dose of toxin. The ordinate FB represents one m.l.d. of toxin. On either side of B points C and D are chosen to represent the limits of experimental error in determining the m.l.d. with a given number of animals. Lines are drawn from points C and D to the origin O cutting Curve 1 at E and H. Then the ratio of the lowest to the highest apparent L+ dose determined with the same number of animals will be as the abscissæ of these points, i.e. as OK is to OL. The value of this ratio depends upon the slope of the curve between the points E and H and approaches nearer to the ratio FD to FC the nearer EH approaches the horizontal. Since no portion of Curve 1 is perfectly horizontal the margin of error in testing an L+ dose is invariably less than that in testing for a fatal dose, a point strangely overlooked by many of the earlier workers in determining the strength of a toxin.

The importance of using a test toxin with a high toxin-toxoid ratio can also be demonstrated. The line NF represents the partial neutralization curve of total antigen and therefore of toxicity if the material under test contained all toxin and no toxoid. The projection of the points of intersection P and R by the lines OC and OD again determine the limits of the observed L+ dose. The angle made between PR and the base line is less acute than that between EH and the base line, and so the margin of error in the determination of the L+ dose decreases with the increase of the toxin to toxoid ratio.

Curve 2 represents another toxin containing less specific toxin, OU, and the same amount, ON, of total antigen. The line joining UF intersects Curve 1 at the point V; then it can be shown that the ratio between

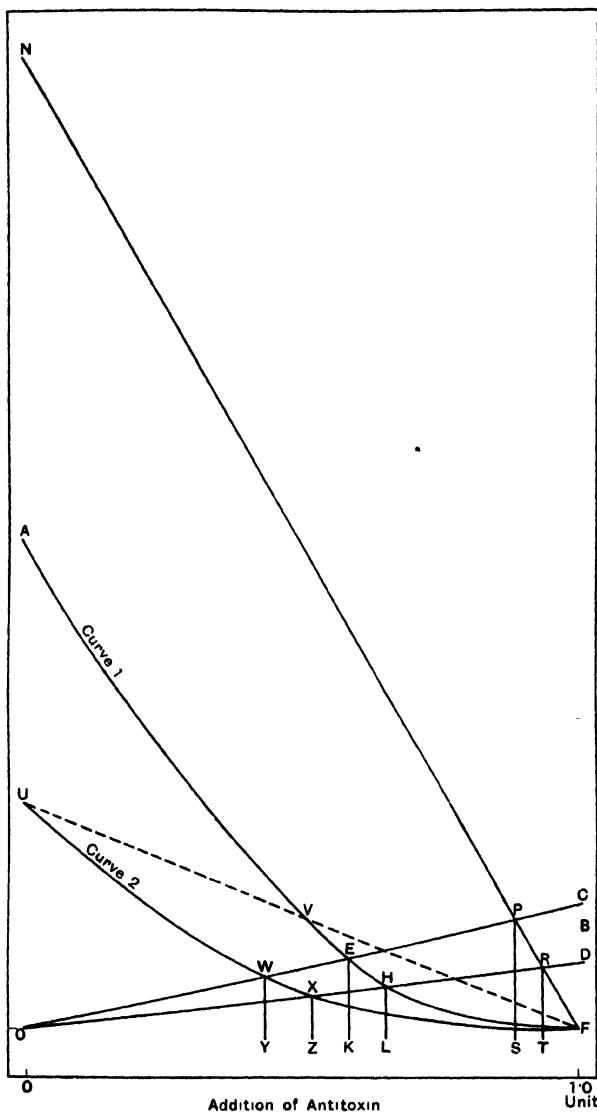


CHART V. Diagrammatic representation of the relation between toxicity and the accuracy of measurement of the L+ dose.

toxin and toxoid in the unneutralized Curve 2 toxin is the same as that left free in Curve 1 toxin when partially neutralized to the stage marked V. The course of neutralization of Curve 2 toxin follows that of Curve 1 toxin from the point V to the base line at F. Curve 2, which has been so plotted, is intercepted by the lines OC and OD at the points W and X.

The projection of these points on the base line again determines the degree of accuracy of testing the L+ dose of such a toxin. Curve 2 is a reproduction of the least steep portion of Curve 1, and therefore the angle made by WX and the base line will be more acute than that made by EH and the base line, and thus the margin of error of testing the L+ dose will be greater. The titration of an unknown antitoxic serum against a chosen test-dose is subject to the same percentage error as the determination of the L+ dose of the same toxin against a fixed unit determined with the same number of animals. If Chart V were correctly drawn to scale, limits of error of titration would be  $\pm 30$  per cent. for the m.l.d., 3 per cent. for the L+ for pure toxin, 6 per cent. for Curve 1 toxin and 11 per cent. for Curve 2 toxin. It must be pointed out, however, that for the sake of clarity of the diagram, the length of FB representing one m.l.d. is greatly exaggerated.

#### DANYSZ PHENOMENON.

A given amount of antitoxin neutralizes less toxin when the latter is added in stages than when it is all added at once. This was first demonstrated with ricin and antiricin by Danysz (1902), who gives the following data :

Single addition of ricin to 0.2 c.cm. antitoxin :

- 0.14 c.cm. produces no reaction (Lo).
- 0.24 c.cm. is just lethal (L+).

Two additions of ricin at 24 hours interval to 0.2 c.cm. antitoxin :

$$0.14 + 0.02 = 0.16 \text{ c.cm. is lethal.}$$

These results were confirmed by von Dungern (1904) using diphtheria toxin and antitoxin. The following figures are typical of the results published by von Dungern :

Single addition of toxin to 1 unit of antitoxin :

- 0.60 c.cm. produces no reaction (Lo).
- 0.74 c.cm. just fails to kill.
- 0.78 c.cm. is lethal to guinea-pigs (L+).

Two additions of toxin at 24 hours interval to 1 unit of antitoxin :

$$\begin{aligned} 0.20 + 0.40 &= 0.60 \text{ is lethal to guinea-pigs.} \\ 0.40 + 0.23 &= 0.63 \quad , \quad , \quad , \\ 0.60 + 0.10 &= 0.70 \quad , \quad , \quad , \end{aligned}$$

Danysz (1902) also showed that an Lo or a slightly under-neutralized mixture appeared to be antitoxic in its action in that a single fatal dose added to an Lo mixture some time later failed to render it toxic ; a mixture of ricin and antiricin killing in 12 days killed in 8 days after the addition of 1 m.l.d. of ricin (killing a guinea-pig in 4 days). From his results, Danysz suggested that a toxin molecule can be either wholly or partially saturated by antitoxin, a theory developed later by Bordet (1903).

Chart VI depicts the Danysz phenomenon graphically.

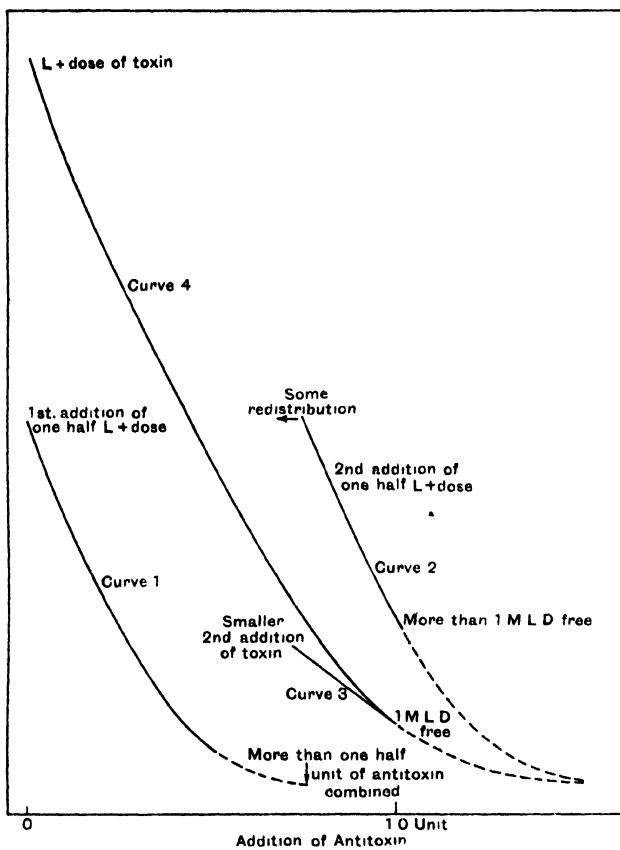


CHART VI. Diagrammatic representation of the Danysz effect.

If only one half the  $L+$  dose of toxin be mixed with one unit of antitoxin more than one half a unit of antitoxin enters into combination. If to this mixture a second addition of one half the  $L+$  dose is made, less than one half per unit of antitoxin is available for neutralization provided there is no redistribution of antitoxin. The course of neutralization of the second addition is that shown by Curve 2 in Chart VI. A much smaller addition of toxin as shown in Curve 3 is sufficient to produce a resulting mixture containing one m.i.d. of toxin free, thus producing the same effect as the single addition of one  $L+$  dose to a unit of antitoxin shown in Curve 4. If there were complete redistribution, as would be the case if the union were a simple chemical reaction obeying the law of mass action, then the toxin in the second addition would replace the excess of toxoid combined and Curve 2 would be shifted to the left and coincide with Curve 4.

From experimental results it is obvious that some redistribution occurs, accounting for the apparent antitoxic effects of the  $Lo$  mixture,

but redistribution is not complete, and so the Danysz phenomenon is produced. Whatever the extent to which the added toxin can turn out toxoid from combination, the total amount of antigen remains the same, and so the Danysz effect should not be obtained in a flocculation reaction. This point is shown graphically in Chart VI, because the curves of neutralization of the full L+ dose and the second addition of one half the L+ dose meet the same point on the base line.

According to Schmidt (1927) the Danysz phenomenon can be demonstrated *in vitro*. This, however, needs confirmation.

The incompleteness of the reaction between toxin and the toxoid-antitoxin complex must mean that either the conditions of the mixtures studied are not equilibrium conditions or that some further slow reaction occurs between toxoid (and presumably toxin) and antitoxin after combination. The second alternative seems the more likely.

The Danysz effect increases with the time interval between the additions of toxin. The alteration in the complex may be only a physical one, analogous to or preceding flocculation, which would result in the formation of large complexes which react slowly. If this were the case the Danysz effect should vanish if the mixture after the second addition of toxin were allowed to stand after mixing and before injection.

#### INTERACTION OF ANTIGEN AND ANTIBODY.

The exact nature of toxin-antitoxin flocculation is not yet known. The toxin-antitoxin reaction may take place in two stages, of which only the second is visible as a floccular precipitate. Or there may be a preliminary combination either 'physical' or 'chemical', and then an additional substance, perhaps lipoidal in character, assists the reaction. Bayne-Jones (1928) suggests that differences in the rate of formation of floccules may be due to qualitative colloidal properties of the fluids or to differences in antigenic potency of the toxins, and the quality of the serum known as its avidity.

The appearance of a floccular precipitate in a balanced mixture implies that the combination of antigen and antibody is insoluble. Mixtures containing a slight excess of either still flocculate, but a large excess of either constituent prevents the formation of any precipitate. According to Bordet, a mixture of toxin and antitoxin does not contain either body in the free state, but the whole of the toxin is said to be adsorbed on to the antitoxin, forming a uniform mixture of partially saturated toxin or partially saturated antitoxin according to the proportions of the two substances present. Whatever the reaction *in vitro*, the effect *in vivo* is that of free toxin or free antitoxin, and the general theory, based upon the curve of neutralization, can still be accepted, but eventually will be modified, in all probability by the substitution of such terms as 'degree of toxicity' in place of 'amount of toxin free'. In like manner, reference to 'the ratio of toxin to toxoid' may be replaced by 'degree of dispersion'.

Glenny and Waddington (unpublished) have shown that addition of excess of antitoxin or toxin to a balanced mixture does not prevent the formation of the Ramon precipitates even though the addition be made long before the appearance of visible flocculation.

The appearance of visible flocculation may not be a simple function of the rate of the preliminary combination of toxin with antitoxin presumed to be loose, and this may bear no simple relation to the rate of ultimate combination, which is assumed to be of a firmer nature. The question of speed of flocculation and difference in the *in-vivo* and *in-vitro* titrations of antitoxin involves the discussion of the important question of avidity.

#### AVIDITY.

Various authors, at different times, have expressed a doubt whether the unit value of an antitoxic serum as determined by the Ehrlich method is a true indication of its therapeutic value. Cruveilhier (1904) concluded that the curative value of the serum determined by the injection into guinea-pigs after the injection of a living culture was independent of its antitoxic value. Thus it was suggested that antitoxic serum might have a qualitative as well as quantitative value. To this qualitative property of antitoxin Kraus gave the name 'avidity'. Glenny (1913) showed that definite qualitative differences were sometimes apparent even by the Ehrlich method of testing, and he described a form of 'modified antitoxin'. Later work by Glenny, Pope and Waddington (1925) showed that this type of antitoxin combined very slowly with toxin and that considerable dissociation occurred when mixtures were diluted. This type of antitoxin of weak avidity can be readily distinguished from the usual type of antitoxin, because it appears to have considerably greater potency when tested by the Ramon flocculation method than when tested *in vivo*.

The therapeutic value of an antitoxin may depend upon the speed with which it will combine with toxin and also upon the firmness of that combination. It is not known whether these two functions are related. Glenny's 'modified antitoxin' can be produced by the action of heat on tricesoled antitoxic serum, but it is also produced naturally by the hyperimmunization of certain horses and of other animals, and has also been found to occur normally in man. As a general, but not universal, rule, the rate of flocculation of modified antitoxin is slow where the *in-vitro* value is greater than the *in-vivo* value. This gives some support to Ramon's claim that the rate of flocculation is a measure of avidity. It appears probable, however, that speed of flocculation depends upon other factors besides avidity. The whole question must be regarded as one of extreme importance needing much investigation. Before any dogmatic statements are made, it is necessary to investigate many samples of antitoxic sera prepared by different workers in various ways. It would appear probable that antitoxin obtained from many horses received from the same general source, and immunized by the same method with toxin which has been prepared in a specified manner, might show a constant

relation between the two quantities, speed of flocculation and rate of combination ; and yet factors appear to exist which disturb such correlation. For example, we have occasionally found rapidly flocculating sera which dissociate *in vivo*, i.e. the *in-vitro* value is greater than the *in-vivo*. If other factors occur in antitoxin produced in other ways, then the method of judging avidity for one type of antitoxin would not be of universal application. The following are the various methods of testing avidity that have been tried or are suggested.

A comparison can be made between the action of mixtures of antitoxin and toxin injected subcutaneously into a guinea-pig and the action of such mixtures injected intravenously into guinea-pigs and rabbits. In the former method of testing, the rate of absorption is sufficiently slow to allow combination to occur between toxin and antitoxin, except in the case of modified antitoxin. Mixtures of modified antitoxin with toxin, injected subcutaneously, produce no local oedema, but guinea-pigs may die within a few days. This suggests that the combination is so loose that the toxin becomes dissociated when the mixture is absorbed into the circulation. The intravenous injection of a mixture does not allow any latent period of combination and reveals degrees of firmness of association.

Madsen and Schmidt (1926) showed that an Lo mixture of a certain toxin with a fast flocculating serum was non-toxic to rabbits when injected intravenously immediately after mixing ; a similar mixture with a slow flocculating serum remained toxic until 14 hours after mixing. The curative values of the two sera were tested by injection intravenously into rabbits one hour after the intravenous injection of 6 m.l.d. of toxin. It was necessary to use 150 units of the slow serum although 10 units of the fast serum was sufficient to save the animal. Locke, Main and Miller (1927) suggest as an avidity unit the amount of antitoxin which neutralizes one Lo dose of the toxin five minutes after mixing, as judged by the intravenous injection into rabbits.

The work so far done on these two methods has dealt with a limited number of sera tested on very few animals, but it appears to bear out Ramon's suggestion that speed of flocculation is a measure of avidity. It is possible, however, that if these authors had tested a fast flocculating serum whose *in-vitro* value was greater than the *in-vivo* value, the results might have been different.

Other methods suggested are the subcutaneous injection of toxin and antitoxin given simultaneously in different places, and the amount of serum to be injected intravenously either just prior or subsequent to the intracutaneous injection of toxin. It would appear advisable also to investigate more fully the Danysz phenomenon both *in vivo* and *in vitro* with wide variation in the time elapsing between successive additions of toxin, and also the effect of adding excess of toxin and antitoxin at different times after the preparation of balanced mixtures. The ease with which toxin-antitoxin floccules can be dissociated, and the differences in the antigenic values of such floccules may also throw some light on the subject.

**Immunity, Antigens and Immunity Response.**

The presence of antitoxin in the blood-stream enables an animal to tolerate toxin to which it is normally susceptible. Such antitoxin may be actively produced in the animal in response to the stimulus given by the administration of an antigen in a suitable form, or may be passively conferred by the introduction of antitoxin produced in some other animal. Active and passive immunity differ in certain important respects. Active immunity is obtained after a definite latent period following the introduction of an antigen and is of long duration ; it is associated with increased power of the animal to respond to further injections of antigen. Passive immunity may be established immediately, but is of relatively short duration ; it leaves unaltered the reaction of the animal to the antigenic stimulus of a specific toxin.

**NATURAL IMMUNITY.**

An active immunity may be naturally acquired in response to sub-liminal infections during life, and a passive immunity may be conferred by placental transmission, or by suckling. Natural immunity in man is of great importance from the public health standpoint, and the presence of natural antitoxin in horses has an important bearing on the production of antitoxin for therapeutic purposes. Abel (1894) was the first to demonstrate diphtheria antitoxin in normal persons and Roux and Martin (1894) in horses.

*Passively Acquired Natural Immunity.*

Ratner, Jackson and Gruehl (1927) have extensively reviewed the literature on maternal transmission of immunity and conclude that the method of transference of antitoxin from mother to young differs in different animals. In man and in rodents there is a single cell membrane separating the maternal and foetal circulation ; antitoxin readily passes and young are born immune. In ruminants the two circulations are separated by three cell-layers through which antitoxin does not pass ; the young are born non-immune. The colostrum of these animals may contain a high concentration of antibodies which is readily absorbed in suckling. Recently Dalling (1928) has shown that lambs born of ewes actively immunized to the toxin of lamb dysentery have no antitoxin at birth, but such antitoxin can be demonstrated within 1½ hours of feeding with colostrum.

Fischl and v. Wunschheim (1895) tested the cord blood of 83 infants at birth and found 83 per cent. contained diphtheria antitoxin, giving an average of 1/5th of a unit of antitoxin per c.cm. of serum. Karasawa and Schick (1910) found more antitoxin in infants at birth than in infants over 6 weeks old. Von Gröer and Kassowitz (1915) found 84 per cent. of 140 mothers and their new-born infants had over 1/200th of a unit of antitoxin per c.cm. of blood. In a large series of observations, v. Gröer and Kassowitz (1919) again found 84 per cent. of infants immune at birth,

but only 32 per cent. immune at the age of 9 to 18 months and 28 per cent. at 2½ to 3½ years old. The immunity rate for the infants at birth corresponded to that of adults of the same population. These observers also showed that passive immunity was maintained far longer in breast-fed than in bottle-fed infants. Sordelli (1920) found diphtheria antitoxin in the blood of 65 per cent. of horses up to 9 months old and none in horses from 1 to 5 years old.

The duration of immunity recorded by different observers depends to some extent upon the delicacy of the methods of testing employed. In the absence of possible additions of antitoxin received by the absorption of milk, the rate of loss of passive immunity, maternally conferred, appears to be similar to that in animals injected with homologous antigenic serum ; thus Glenny and Hopkins (1923<sup>2</sup>) showed that guinea-pigs bred from an actively immunized mother lost antitoxin at the rate of 25 per cent. a week, and reached 1/10th of their original antitoxic content in 8 weeks from birth. Horses injected with diphtheria antitoxin actively produced in other horses, lost their passively conferred immunity at the rate of 20 to 23 per cent. a week, and reached 1/10th of their original antitoxic content in from 8 to 12 weeks after injection.

Anderson (1906) and Theobald Smith (1907, 1909) demonstrated considerable immunity in the young of guinea-pigs injected with a toxin-antitoxin mixture. It is important, therefore, for test purposes to avoid using laboratory animals bred from mothers previously used for tests upon the same species of toxin.

#### *Actively Acquired Natural Immunity.*

Natural immunity in other than very young animals is associated with an increased power of antigenic response, and is without doubt actively acquired in response to antigenic stimulus. Virulent *C. diphtheriae* may be found in the throats of healthy animals and cutaneous or wound diphtheria is not uncommon. Conditions of life favouring repeated contact with *C. diphtheriae*, age, density of population and contact with known cases, increase the immunity rate ; carriers frequently have a high antitoxic content. *C. diphtheriae* has rarely been found in the throats of horses, the only recorded instances being those of Cobbett (1899<sup>1</sup>) and Ramon (1925). A more frequent focus of infection appears to be in wounds. Cases have been described by Minett (1922), Kliewe and Westhues (1925) and by Parish and Okell (1926). The author, in conjunction with his colleagues, has demonstrated a considerable rise in antitoxic content when a horse with a superficial wound becomes infected with *C. diphtheriae*.

Glenny (1925<sup>1</sup>) referred to unconfirmed work suggesting the occurrence of natural antitoxin in the blood of cats. The author has recently found antitoxin in the blood of 5 out of a herd of 84 cows. Numbers of guinea-pigs and rabbits have been examined, but there is no recorded case of naturally occurring antitoxin. It is suggested that more extensive examination of the blood of domestic animals may throw further light

on the part that animals play in the spread of diphtheria. The carrier state may be transient, but the resulting antitoxic content of long duration.

TABLE V.  
Showing the Factors influencing Natural Immunity.

MEN.						
<i>Proportion Schick-Negative.</i>						
<i>Age</i> .. ..	Months.	Years.	Years.	Years.	Years.	Years.
	0 to 6	½ to 3	3 to 5	5 to 10	10 to 20	Over 20
	85	40	60	70	80	88 Park.
	per cent.	per cent.	per cent.	per cent.	per cent.	per cent.
	(passive.)					
<i>Density of Population</i> ..	Isolated.			Crowded.		Zingher.
	30 to 50 per cent.			75 to 84 per cent.		
<i>Contact</i> .. ..	No exposure.	One epidemic.	Two epidemics.	Dudley.		
	60 per cent.	80 per cent.	95 per cent.			

HORSES.								
<i>Proportion containing Antitoxin.</i>								
<i>Age</i> .. ..	Months.	Years.	Years.	Years.				
	0 to 9	1 to 2½	3 to 5	10 to 17				
	65 per cent.	0 per cent.	0 per cent.	100 per cent.	Sordelli.			
	(passive.)							
<i>Density of Population</i> .. ..	After 1917.			Before 1917.				
	(1/10th unit or more)							
	35 per cent.			70 per cent.				
<i>Contact</i> .. ..	On arrival in camp.			After 9 days in camp.				
	(1/25th unit or more)							
	37 per cent.			100 per cent.				

Table V published by Glenny (1925<sup>1</sup>) summarizes factors influencing the immunity rate in man and in horses. The figures given of the immunity rate at different ages in man are those of Park (1921). Similar figures have been obtained by v. Gröer and Kassowitz (1919) and by numbers of other workers. Zingher (1921) demonstrated the effect of density of population on the immunity rate. Dudley (1922) showed that in a school that had had two distinct outbreaks of diphtheria, 95 per cent. of the boys exposed to both epidemics were immune compared with 85 per cent. exposed to one epidemic, and 60 per cent. of those who had entered the school after the last epidemic. O'Brien (1923) found 86 per cent. immune among boys of a training ship in which diphtheria had been prevalent; among incoming boys only 57 per cent. were immune. Sordelli (1920) found all horses over 10 years of age to be immune. Glenny (1925<sup>2</sup>) tested

the antigenic content of the blood of 1,350 horses over 7 years of age, and found more than 1/10th of a unit of antitoxin per c.cm. in 70 per cent. of those tested between the years 1907 and 1915, and in 35 per cent. of those tested after 1917. He suggested that this difference was due to the greatly lessened number of horses in towns.

Glenny (1925<sup>2</sup>) demonstrated frequent fluctuation in the antitoxic content of normal horses and suggested that this was evidence to show that in large stables many horses have some stimulation, on an average once every two months. It must be pointed out that fluctuation in the natural immunity of man may not be due to subliminal infection, if the subjects have been Schick-tested previously, as it has been shown (see p. 174) that Schick toxin itself is sufficient to produce an antigenic stimulus. Dudley (1929) has summarized very completely the present state of our knowledge with regard to the use of the Schick test in detecting natural immunity and the effect of exposure to infection upon the increased immunity rate of the population.

#### PASSIVE IMMUNITY.

The rate at which an animal is passively immunized depends upon the method of administration, but the duration of passive immunity depends on the relation between the species of the donor and the recipient of the antitoxin, and upon the degree of sensitization of the animal to the antitoxin protein.

##### *Method of Administration of the Antibody.*

Henderson Smith (1907) showed that after the intraperitoneal injection of antitoxic serum, the antibody in the blood does not reach its maximum until 25 to 30 hours later ; after subcutaneous injection, the interval is from 2 to 3 days and by intravenous injection, the maximum amount is obtained at once. After the subcutaneous injection of 9,000 units of diphtheria antitoxin into a man weighing 72 kilos, he found the following amounts of antitoxin in the blood :

5 hours after injection	0·1	of a unit per c.cm.
14 "	0·22	" "
32 "	0·68	" "
44 "	1·0	" "
3 days	1·13	" "
4 "	1·13	" "

The rate of absorption of antitoxin in man appears to correspond very closely with that in other animals. Table VI gives Henderson Smith's figures for man in terms of the percentage of the maximum titre reached. Similar figures are given in the same table for the antitoxic content of a rabbit, sheep and goat after the subcutaneous injection of antitoxin, from the results of Glenny and Hopkins (1923<sup>2</sup>) ; the figures given for goat are slightly too high, because the percentages are based upon the amount present four days after the injection, which is probably lower than the maximum, as a third day reading was not taken.

TABLE VI.

Antitoxic titre of blood after the subcutaneous injection of diphtheria antitoxic serum obtained from a horse, given in terms of the percentage of the maximum titre reached in 2 to 3 days.

Hours after Injection.	Man.	Rabbit.	Sheep.	Goat.
1	..	1	—	—
2	..	1½	3½	—
3	..	4½	—	—
4	..	8	7½	10
5	..	12	—	—
6	..	15	11	17
8	..	25	—	—
14	20	—	—	—
24	—	60	50	66
32	60	—	—	—
48	88	100	80	92
72	100	—	100	—

It will be seen from Table VI that 4 to 6 hours elapse after the subcutaneous injection of antitoxic horse-serum before the blood shows 1/10th of the maximum amount which is present 2 to 3 days later. At the end of 24 hours about one-half or two-thirds of the maximum is present.

A comparison between intravenous, intramuscular and subcutaneous injection of antitoxin is given in Table VII from results partly published by Glenny and Hopkins (1923<sup>1</sup>). These authors state that following a subcutaneous injection, very little antitoxin is absorbed during the first two hours, but after that about 1 per cent. of the total injected is absorbed per hour for the next eight hours. Following an intramuscular injection, absorption occurs earlier, and after an intravenous injection, the full amount of antitoxin is immediately distributed throughout the circulation. From Table VII it is seen that two hours after the intravenous injection of antitoxin, there is present in the blood more than 200 times the amount of antitoxin present 2 hours after a subcutaneous injection. For the same interval of time there is present more than twice as much antitoxin after intramuscular than after subcutaneous injection. Three days after the injection, the same amount of antitoxin is present in the blood whichever method of administration is adopted.

Morgenroth and Levy (1912) obtained different results after injecting 13 rabbits subcutaneously, intramuscularly and intravenously. Five hours after injection, the intramuscular showed up to 15 times the amount of antitoxin obtained by the subcutaneous injection.

TABLE VII.

Comparison between antitoxin content of blood of rabbits after intravenous, intramuscular and subcutaneous injection of the same quantity of diphtheria antitoxin.

Hours after Injection.	Antitoxic Content in Terms of Percentage of that present at same time after Intravenous Injection.	
	Intramuscular.	Subcutaneous.
2 ..	1·06	0·47
4 ..	4·3	3·2
6 ..	11·0	6·5
24 ..	50·0	42·0
48 ..	77·0	86·0
72 ..	100·0	100·0

#### *Duration of Passive Immunity.*

Antitoxin is always associated with the specific serum protein of the animal in which the antitoxin has been produced ; the duration of immunity depends, therefore, on the rate at which the animal eliminates the foreign protein introduced. Homologous proteins are eliminated slowly (passive immunity of infants through maternal transmission lasts six months or more) ; heterologous serum is rapidly eliminated, and passive immunity acquired by the injection into man of antitoxin obtained from a horse is lost in three weeks or less. When the species from which the serum has been obtained is closely related to that of the animal injected, an intermediate rate of elimination occurs.

Glenny and Hopkins (1922) and (1923<sup>1,2 & 3</sup>) studied the fate of both homologous and heterologous diphtheria antitoxic serum injected into a number of animals ; more detailed work concerned horse-serum injected into rabbits. They found that the rate of loss of heterologous antitoxin can be divided into three phases, varying in their duration with the condition of the animal in relation to the serum injected.

#### *In normal animals.*

*Phase A.* An initial fall of 50 per cent. in antitoxic content of the blood occurring within 24 hours and often within 6 to 8 hours ; the suggested explanation is that this fall is due to antitoxin distributed from the blood to the tissues. Later work by Glenny and Waddington (1929) lends support to this view.

*Phase B.* A gradual constant percentage loss of approximately 25 per cent. from day to day, lasting usually 6 to 7 days. This phase of gradual loss appears to be due to the elimination of foreign protein by some method of metabolism.

*Phase C.* A rapidly accelerated loss of 50 per cent. or more per day due to the active production of precipitin as a response to the injection of foreign protein. Longcope and Rackemann (1918) showed that when horse-serum is injected into man there is a sudden loss of antigen (proteins) corresponding to a rapid production of precipitin. It sometimes happens that the amount of precipitin formed is insufficient to eliminate all the protein injected, and phase C is followed by a gradual loss corresponding again to phase B.

*In sensitized animals* (i.e. those potentially immune to serum protein).

*Phase A.* A 50 per cent. fall as in normal animals.

*Phase B.* A gradual loss at the same rate but of shorter duration than that of normal animals.

*Phase C.* Extremely rapid elimination resulting in almost total loss in 24 hours.

When homologous serum is injected, phase A loss occurs, and the rate of loss corresponding to phase B is much slower, about 20 to 25 per cent. per week, continuing until available methods of testing can no longer detect antitoxin.

In animals immune to serum protein, phase A is entirely masked by a very rapid elimination due to the presence of circulating precipitin. After the fourth injection of horse-serum intravenously into one rabbit, only 1/400th of the antitoxin injected could be detected within 15 minutes of the injection. After the subcutaneous injection of antitoxin into another rabbit, the maximum amount of antitoxin appearing in the blood was only 1/15,000th of the total antitoxin injected.

A general consideration of these results shows that precipitin is a result of the injection of a foreign protein and follows the general law of antibody production. No extensive work has been done to show the extent to which human subjects may vary in their degree of immunity (sensitization) to horse protein.

#### ACTIVE IMMUNITY.

The injection of an antigen is followed by the appearance of antitoxin in the circulation of the animal and the animal acquires an increased power of response to subsequent injections.

A single injection of toxin confers a very low degree of basal immunity, the amount of antigen injected being of necessity very small. Theobald Smith (1907) showed that neutral mixtures of toxin and antitoxin were antigenic; toxoid affords another method of administering large doses of antigenic material. By means of these two products, the general principles of immunity response, long recognized, have been studied more

recently in greater detail. Glenny and Südmersen (1921) studied the rate of immunity response in guinea-pigs surviving mixtures of diphtheria toxin and antitoxin given in the routine testing of sera for antitoxin content. They compared the rate of response with that following a second injection into the same guinea-pigs, and from these results, confirmed on other animals, they showed that there is a considerable difference between the response to an initial dose given to a normal animal and that given after basal immunity has been established. They distinguished between these two types of response by the terms 'primary response' for the result of the first primary stimulus in an animal and 'secondary response' for that following the second stimulus, i.e. that given to an animal already immune. This fundamental difference between the primary and secondary response was established for guinea-pigs, rabbits, goats and horses. The same differences have since been established by Glenny (1925<sup>1</sup>) for tetanus toxin and toxins of three gas gangrene organisms, *B. welchii*, *Vibrio septique* and *B. cædematiens*.

The difference between primary and secondary response shows that an injection of an antigen into a normal animal is followed not only by

TABLE VIII.

Showing the Comparison between Primary and Secondary Stimulus Response.

	Primary Stimulus (Normal Animals).	Secondary Stimulus (Immune Animals).
<b>Latent Period :</b>		
Average .. .. .. ..	3 to 4 weeks	3 to 4 days.
Minimum .. .. .. ..	9 days	2 days.
<b>Time of Maximum Antitoxic Content :</b>		
Average .. .. .. ..	2 to 3 months	8 to 12 days.
<b>Antitoxic Response :</b>		
Average .. .. .. ..	0.1 unit	1 to 10 units.
Maximum (Rabbits) .. .. ..	0.26 unit	65 units.
Maximum (Guinea-pigs) .. ..	1.5 units	80 units.
<b>Size of Stimulus to Produce Potential Immunity.</b>	1/100 to 1/10 Lf	—
<b>Size of Stimulus to Produce Detectable Antitoxic Response.</b>	1/10 to 1 Lf	1/1,000 Lf or less.

the production of an antibody, but also by increased ability to respond to subsequent injection. This condition of 'potential immunity', Glenny (1925<sup>1</sup>) has also been termed by Zoeller (1925) 'réactivité acquise'.

Glenny and Allen (1922<sup>1</sup>) showed that the injection of an antigen into an animal may be followed by an increased power of response before any circulating antitoxin can be detected, and further, the condition of 'active immunity' continues after the disappearance of circulating antitoxin produced by an animal in response to a previous stimulus. The injection of an antigen into a normal animal is always followed by a 'latent period' before any antitoxin appears in the blood; if the animal is actively immune, there is still a latent period before any increase in antitoxin occurs. The essential difference between primary and secondary stimulus response lies in the duration of the latent period and in the amount of antitoxin produced.

Table VIII summarizes the essential differences between the two types of response.

#### *Primary Stimulus.*

Glenny and Südmersen (1921) tested about 350 guinea-pigs that had been used for routine testing of antitoxin against one test-dose of toxin by subcutaneous injection. The test guinea-pigs were subsequently injected, after various intervals of time, with different volumes of toxin. Table IX is a summary of their results.

TABLE IX.

Showing the average number of m.l.d. of toxin tolerated by guinea-pigs previously injected with mixtures of antitoxin and one test-dose of toxin.

Interval of Time between Primary Stimulus and Subsequent Test.	No Local Edema (Lo or Over-neutralized Mixtures).	Local Edema (Lo to L+ Mixtures).
2 weeks .. .. .. .. ..	1	1
3 .. .. .. .. ..	2	3
4 .. .. .. .. ..	13	13
6 .. .. .. .. ..	40	100
8 .. .. .. .. ..	100	400
12 .. .. .. .. ..	—	400
16 .. .. .. .. ..	200	—

Some guinea-pigs injected with mixtures causing local reaction were bled 8 to 12 weeks later and their blood tested for antitoxin. Out of 7 guinea-pigs so tested, 3 contained 0.14 unit per c.cm., 2 less and 2 more. This level of antitoxic content corresponds, therefore, to a degree of tolerance supporting 400 m.l.d. (6.25 Lo doses of the toxin used). The

highest antitoxic content recorded as a result of a single primary stimulus was 1·5 units per c.cm., 12 weeks after the injection of 25 Lo doses of toxin into a guinea-pig passively immune by maternal transmission.

When a greater primary stimulus is given, such, for example, as an injection of unneutralized toxoid, a higher degree of tolerance may result. Ramon (1924<sup>1</sup>) states that guinea-pigs injected with 1·0 c.cm. of 'anatoxine' survive several fatal doses of toxin 15 to 18 days after the original injection and from 50 to 100 m.l.d. at the end of a month.

The latent period between the primary stimulus and the appearance of immunity in the animals recorded by Glenny and Südmersen was usually of 3 weeks' duration, occasionally as short as 2 weeks or as long as 9 weeks. Subsequent experiments with more powerful antigens, toxoid or concentrated toxoid, have reduced the latent period to 10 days in a number of guinea-pigs and to 9 days in several rabbits (Glenny, Hopkins and Pope, 1924).

#### *Secondary Stimulus.*

An animal that has already responded to a primary stimulus and produced antitoxin can tolerate many times the dose of toxin that would be fatal to a normal animal. The increased response to a secondary stimulus, however, is not explained by the increased dose that can be given to the animal. Glenny and Allen (1922<sup>2</sup>) showed that a dose of toxin as small as that used in the Schick test may act as a secondary stimulus. In one example quoted by them, a rabbit which had previously received a primary stimulus but had not produced any detectable antitoxin, i.e. less than 1/2,000th of a unit per c.cm. of blood, produced 1 unit of antitoxin per c.cm. in 7 days after the intracutaneous injection of a Schick dose of toxin.

Glenny and Südmersen (1921) reported that 12 guinea-pigs that had been injected with various toxin-antitoxin mixtures and had produced antitoxin to the extent of 0·25 unit per c.cm. or less, 8 to 12 weeks later were injected with 400 m.l.d. of toxin; 8 out of 12 produced over 20 units per c.cm. within 2 weeks of injection.

An Lo mixture of toxin and antitoxin given as a secondary stimulus 3 months after a primary stimulus of a similar mixture, caused the production of 20 units of antitoxin per c.cm. in 2 guinea-pigs out of 5. Seven rabbits were given 2 c.cm. of toxin (6 Lo doses) 9 to 16 weeks after their primary stimulus; all produced over 20 units, and 4 of the 7 over 50 units, of antitoxin per c.cm. 8 to 12 days after the stimulus.

The increased power of response conferred by a primary stimulus lasts for a long period, and there is no recorded case of its diminution. Glenny and Südmersen state that the pooled serum of a number of guinea-pigs given a secondary stimulus of toxin 9 months after their primary stimulus yielded 30 units of antitoxin per c.cm. Another guinea-pig injected 22 months after a series of preliminary injections gave over 20 units per c.cm. The latent period after a secondary stimulus

was never more than 5 days, and the maximum antitoxin content was reached in 8 to 12 days. A sufficient interval of time must elapse after the primary stimulus for potential immunity to be established fully, if the secondary stimulus is to produce a maximum response.

Glenny and Südmersen have shown that naturally immune horses and men give a secondary response to the injection of an antigen, showing that their immunity has been actively acquired. The injection of toxin or toxoid into a horse with normal antitoxin is always followed within a few days by an increase in antitoxin content ; on the other hand, a horse without detectable antitoxin (less than 0·0005 unit per c.cm.) may yet be potentially immune and give a secondary response to an antigenic stimulus while others are non-immune and give only a primary response. The author observed that one horse with a normal antitoxic content of 0·5 units contained 40 units per c.cm. 7 days after the injection of 2 c.cm. of toxoid. Ramon (1924<sup>2</sup>) has recorded that one horse produced 300 units per c.cm. as a result of a single injection of 25 c.cm. 'anatoxine'.

#### *Secondary response to natural stimuli.*

Glenny (1925<sup>2</sup>) tested the normal antitoxic content of 131 horses immediately after purchase and again after an interval of at least 1 week ; 26 per cent. showed a higher normal value on the second occasion. One horse with 0·1 unit of antitoxin per c.cm. of blood contained 10 units 9 days after arrival and 20 units 5 days later, without receiving any injection. Further instances are given by Glenny (1925<sup>1</sup>) of 8 horses in a military camp suffering from slight abrasions. When tested 9 days later, all showed an increased antitoxic content ; 1 horse containing less than 0·0005 unit when first tested increased in antitoxic content to 8 units.

On a later occasion, horses in another camp were tested for antitoxic content ; of 18 horses with slight abrasions, only 1 with an initial value of 0·01 unit per c.cm. showed any increase in antitoxic value 10 days later. This horse increased in antitoxic titre to 5 units and virulent *C. diphtheriae* was isolated from the wound. Swabs of the wounds of the other 17 horses were negative (Glenny, Okell and Parish, unpublished). It is generally accepted that the maintenance of immunity in the general population is due to repeated antigenic stimuli given by slight infections.

#### *General Principles of Immunity Response.*

A knowledge of the essential differences between primary and secondary stimulus response is necessary for the successful immunization of animals or of man. In the hyperimmunization of horses against toxins to which they have no natural antitoxin, such as those of most of the pathogenic anaerobes, and of dysentery, it is advantageous to give a few injections to act as a primary stimulus and then to rest the horse until potential immunity develops, and subsequent injections given a month or more later are followed by secondary response. In this way, the general condition of the animal is preserved by giving only such injections as are of maximum antigenic value.

For the immunization of children, it is customary in England and America to give three weekly injections of suitable antigens. This procedure has no scientific foundation, but has apparently been adopted for convenience of administration. The Bacteriological Committee of the Medical Research Council (1923) suggests that the interval between the first and the second injection should be longer than two weeks. The French workers allow an interval of three weeks to elapse between the first and second injection. If the interval is sufficiently long to allow potential immunity to develop, then the second injection acts as a secondary stimulus and a rapid production of antitoxin follows.

*Variation in antigenic response.*

Apart from variation between individual animals, the immunity response to a given injection of antigen depends, within limits, upon the extent to which potential immunity has developed. Successive stimuli are followed by quicker and greater production of antitoxin.

An actively immune animal, that is, one with circulating antitoxin, always exhibits a definite response ; there are degrees, however, of the

TABLE X.  
Showing the Degrees of Immunity in a Normal Population.

Schick Test.	1.	2.	3.	4.	5.
	Negative.	Positive.	Positive.	Positive.	Positive.
Antitoxin in the Blood	Over $\frac{1}{30}$ th unit per c.cm.	Under $\frac{1}{30}$ th unit.	None	None	None.
Response to Injection	Secondary	Secondary	Secondary	Inter- mediate.	Primary.
Earlier Stimulation ..	Many	Many	Many	Few	None.
Response to Immunization.	—	Rapid	Rapid	Slower	Slowest.
Description ... .	Schick- negative immune.	Schick- positive immune.	Potentially immune.	Sub- immune.	Non- immune.

extent to which a potentially immune animal may respond and there must be all shades of partial or sub-immunity. In human practice, a further division of the population is made by means of the Schick test, which divides the actively immune into two groups: those above and those below a certain level of antitoxic content, said to be about 1/30th unit per c.cm. This figure is probably over-estimated; Schick, v. Gröer and Kassowitz (1924) give the level of the Schick test as 1/200 antitoxic unit per c.cm. The five groups into which a population can be divided are shown in Table X.

This grouping is mainly of theoretical interest, because the frequent exposure to subinfective doses of diphtheria causes a rapid transition through Groups 4 and 3 to Groups 2 or 1.

If we are right in assuming that natural immunity in man and in animals is caused by repeated stimulations with small doses of toxin produced by small transient infections, then in any chosen group of children subject to the same general conditions, the number who are already Schick-negative must be an index of the number and extent of infection and stimulation. It follows, therefore, that if in any group there are many people falling into the first division of Table X, there will be a correspondingly large number of Schick-positives in the second and third division, all of whom will respond to artificial immunization so rapidly that they will become Schick-negative in a few weeks, and of those who are apparently non-immune, more will be in the fourth than in the fifth group. There is thus a close connection between the Schick-negative rate of the population before artificial immunization and the ease with which they will become immunized. Zingher (1922), who compared the immunizing effect of certain toxin-antitoxin mixtures upon the children of different schools, showed that the schools with a high natural immunity rate were more easily immunized than those with a low initial rate. Thus, it is evident that the natural immunity rate must always be considered when the efficiency of any protective measure is being judged.

#### *Individuals refractory to immunization.*

A certain proportion of adults remain non-immune although they have been exposed throughout their life to infection on many occasions. Two alternative explanations offer themselves: either some individuals possess another line of defence against invasion, so that they never receive antigenic stimulation of diphtheria toxin, or they give no antigenic response to small doses of toxin. It has been found that Schick-positive adults are difficult to immunize. Schick (1923) has said that 'we believe that there are individuals who are totally incapable of developing antibodies against diphtheria'. If non-immune adults are difficult to immunize solely because they fall into Group 5, and no invading organisms have produced any toxin to render them 'subimmune', then very young children who have had little opportunity of acquiring small stimuli should also be difficult to immunize, but this is not so.

Similar results are seen among horses. In the author's experience, a horse with natural antitoxin will usually, but not invariably, produce antitoxic serum of higher value than a horse that needs an artificial stimulus to establish basal immunity. Variations in horses as antitoxin producers may depend to some extent upon the number of natural stimuli they have previously received. Of the many thousands of horses that have been used for the production of antitoxin, there is no recorded case of any one failing to produce some antitoxin, but many produce very little however they are immunized.

Hiss and Atkinson (1900) have shown that the pseudo-globulin content of the blood rises during immunization. Ledingham (1907) reported that a horse producing an average amount of antitoxin increased greatly in pseudo-globulin content, but another that failed to produce more than half the average amount had a very high pseudo-globulin content to start with. The differences between these two horses are shown in Table XI.

TABLE XI.  
Protein Content of Horse-Serum in Relation to Immunization.

	Total Protein in 10 c.cms. Serum.	Pseudo-globulin in 10 c.cms. Serum.	Percentage Pseudo-globulin of Total Protein.
Horse difficult to Immunize : Normal Content .. ..	0·8532	0·7191	84 per cent.
Horse of Average Ability : Normal Content .. ..	0·8266	0·4365	52 ..
After Immunization .. ..	0·9387	0·6893	73 ..

Ledingham's results suggest that the failure of an individual or an animal to become immune may be connected in some way with a temporary or permanent condition of their blood. That this condition may be hereditary is suggested by some results recorded by Hirschfeld and Brokman (1924), who examined the Schick reactions of 105 children from 42 families. They showed that: (1) When both parents were Schick-positive, all the children were Schick-positive; (2) When both parents were Schick-negative, most of the children were negative, but some young children were positive; (3) When one parent was Schick-negative and the other positive, children of the same blood group as the Schick-positive parent were also positive, while those of the same blood group as the negative parent were mostly negative, but a few were positive.

If it is assumed that all except the young children, who formed exceptions to the general rule, had received reasonable chances of antigenic stimulation, then the Schick-positive members of any family must have differed from those who were negative in being more refractory to immunization. There is no evidence that blood grouping *per se* has any correlation with the Schick reaction; it appears probable, however, that ease of immunization may be hereditary and transmitted along the same lines as blood grouping. This was suggested by Theobald Smith (1909) when he stated in reference to the transmission of immunity that if the father 'exerts any influence upon his offspring, it must be in transmitting a greater or lesser capacity for the production of antitoxin'.

Hirschfeld and Brokman (1924) suggest that 'the greater percentage of immune individuals among urban poor people need not all be an expression of the acquired immunity of the generation in question, but can depend upon a selection of immune individuals in preceding generations'.

#### *Methods of administration of antigens.*

No great differences have been established between the immunity response following the administration of an antigen by subcutaneous, intramuscular, intracutaneous or intraperitoneal injection. Immunity may result from intrathecal injection, but it is of little practical interest. Mutermilch and Salamon (1929) claim that after the intrathecal injection of tetanus toxoid guinea-pigs can tolerate tetanus toxin given either intramuscularly or intrathecally, but after intraperitoneal injection of the toxoid there is intramuscular but not intrathecal tolerance. Glenny and Pope (1925) show that the intravenous injection of diphtheria toxin-antitoxin mixtures produces a primary stimulus response in normal rabbits. Unneutralized toxoid injected intravenously is less effective than the same toxoid partially neutralized by antitoxin; the presence of the antitoxin may assist in delaying absorption or elimination. The intravenous injection of toxin into an animal already immune will not produce a secondary stimulus response if the total circulating antitoxin is sufficient to neutralize more than 50 times the amount of toxin injected. If the amount of toxin injected intravenously is sufficient to combine with 10 to 20 per cent. of the total antitoxin present, as great a secondary response may occur as that which follows a subcutaneous injection. It is obvious, therefore, that intravenous injection is useless for the hyper-immunization of horses which in very early stages of immunity contain a total of over a million units of antitoxin. It is interesting to consider the fate of toxin injected into such animals. A rise in antitoxic value of a horse already containing 1,000 units of antitoxin per c.cm. of serum may follow a subcutaneous injection of 100 c.cm. of toxin, a quantity which could be fully neutralized by 1 c.cm. of serum. In such a horse toxin injected subcutaneously can still act as an antigen when there is enough antitoxin in the total circulation to neutralize 30,000 times the amount

of toxin injected. Does toxin act as a local stimulus, or is it carried in some form to the seat of antitoxin production through the barrier of an overwhelming amount of antitoxin? It may be that the local tissues alter the toxin in some way so that it is no longer readily neutralized by antitoxin and does not suffer the same fate as toxin introduced directly into the circulation.

Attempts have been made to immunize children by oral administration. Reiter and Soldin (1926) claim to have succeeded with toxin-antitoxin mixtures given by mouth after sodium benzoate. Dold and Weyrauch (1927) failed to immunize a group of fifty rabbits by giving orally toxin or toxin-antitoxin mixtures with or without sodium benzoate. Brazil and Vellard (1927) demonstrated in guinea-pigs and rabbits that toxin is absorbed by the mucous membrane of both mouth and nose, and recommended nasal application of 'anatoxine'. Lesné, Marquezy, Lemaire and Monmignant (1927) successfully immunized children by this method, while Copeman (1927) produced immunity by spraying the throat of children with toxin-antitoxin mixtures.

#### *Non-specific factors.*

The amount of antibody produced in an animal as a result of the injection of a given amount of antigen depends upon many factors in addition to the degree of immunity already present in the animal injected. Immunity response to one antigen may be lessened or repressed by active production of another antibody, by blockade of the cells of the reticulo-endothelial system or by a general lowering of the health of the animal. It also appears possible that the presentation of an antigen in such a form and in such a manner that it can be rapidly eliminated will not result in immunity response. Immunity response to an antigen can be increased by the addition of a number of non-specific substances such as turpentine, toluol, tapioca and alum. Finally the injection of certain salts, notably manganese chloride, may produce an increase in circulating antitoxin in animals actively immune.

It is well established that there are qualitative differences in batches of toxin and of toxoid; different preparations of antigens or antigens modified in different ways do not necessarily produce the same response in an animal. The amount of specific diphtheria toxin or antigen is very small in relation to other materials present in a broth culture filtrate. The other substances present may include bacterial proteins in addition to certain altered and unaltered constituents of the original broth, which may act as antigens, and if present in excess in certain batches may lessen the immunity response to the specific toxin. Other substances present in varying amounts in different batches, or present in some and absent in others, may tend to lower the health of the animal, or, on the other hand, act as local irritants only and increase immunity response. The rate at which the toxin can be absorbed may vary with the non-specific substances present in different batches. Finally there may be qualitative differences

in the pure antigen present ; in the present state of our knowledge and in the absence of any method of isolating the pure antigen, it is not possible to determine whether any given difference in response is due to differences in the specific antigen or in the surrounding medium.

The composition of diphtheria toxins may be shown thus :

	<i>Relative Proportions.</i>	<i>Contents.</i>	<i>Reaction.</i>
Toxin (broth filtrate).	99 per cent. = non-specific material.  1 per cent. = specific antigen.	Antigenic material ..  Pseudo - constituent (bacterial protein).  General broth material..	Interference in immunity response.  Severe reactions in certain individuals (mostly adults).  Harmful or advantageous
		Toxin. Present in approximately equal parts in fresh filtrates : on keeping toxin changes to toxoid, or it can be completely changed to toxoid by the action of formaldehyde and certain other agents.	Antigenic : combines with antitoxin : causes oedema and death.
		Toxoid.	Antigenic : combines with antitoxin : causes no ill effects.

*'Crowding out' of immunity response.* The active production of antibody to one antigen lessens the production of antibody to a second antigen. Benjamin and Witzinger (1912) showed that preliminary injection of horse-serum inhibited the formation by rabbits of haemolysin for sheep cells. Lewis (1915) showed that 'an amount of protein that will produce a marked anaphylactic sensitization when injected alone into a guinea-pig will fail to do so if injected together with, or 24 hours after, a much larger amount of another protein'. According to Lewis, these results may be explained by the conception that the numbers of receptors in the body that can unite with a foreign protein is limited. The inhibiting protein, if present in large amounts, combines with all or almost all of these receptors.

Glenny, Hopkins and Waddington (1925) showed that the injection of diphtheria toxin-antitoxin mixtures or of toxoid into immune rabbits caused a small or no antitoxic response if given at a time when the rabbits were producing precipitins to horse-, goat- or cow-serum. They also showed that the active production of precipitin to horse-, goat- or cow-serum interfered with a primary response to diphtheria toxoid. Glenny and Waddington (1928) have demonstrated non-specific interference between one antigen and another in guinea-pigs by means of the 'immunity index method'.

The 'crowding out' of immunity response has been definitely established when one antigen is greatly in excess of another ; in 1·0 c.cm. of horse-serum about 60 mgm. of presumably antigenic material is present ; in 1·0 c.cm. of toxoid the specific diphtheria antigenic content is probably far less than 1 mgm. Glenny and Waddington (1926) showed on a limited number of animals that in a combined 'diphtheria scarlet-fever prophylactic' an excess of scarlet-fever toxin depresses the immunization effect of the diphtheria prophylactic.

Huntoon and Craig (1921) state that 'it is undoubtedly true in many instances that attempts at multiple immunization fail to produce adequate antibody response'. The author has never succeeded in obtaining a simultaneous response to more than three toxins. Several horses with a ground immunity to tetanus, *B. perfringens*, *Vibrio septique* and *B.ædematiens* toxins were injected with a mixture of all four toxins ; in each instance there was failure to respond to one of the toxins. Several horses were so immunized as to contain circulating antibodies to the four toxins, but in no instance were these antibodies simultaneously produced nor present in considerable amounts. Many horses were simultaneously immunized against two or three toxins, those of tetanus, *B. welchii* and *V. septique*. Glenny (1925<sup>2</sup>) reports that in a group of horses the highest value serum produced contained :

- 1,000 tetanus units (American) when immunized alone.
- 600 tetanus units (American) when immunized with one other toxin.
- 400 tetanus units (American) when immunized with two other toxins.
- 10,000 *V. septique* units (provisional war) when immunized alone.
- 10,000 *V. septique* units (provisional war) when immunized with one other toxin.
- 3,000 *V. septique* units (provisional war) when immunized with two other toxins.
- 5,000 *B. welchii* units (provisional war) when immunized alone.
- 4,000 *B. welchii* units (provisional war) when immunized with one other toxin.
- 1,000 *B. welchii* units (provisional war) when immunized with two other toxins.

It must be borne in mind, however, that as Huntoon and Craig (1921) have pointed out, 'failure to produce an antibody response . . . can be attributed to . . . factors influencing the general condition of the animal and indirectly the immunity mechanism'. In attempts to hyperimmunize a horse to many toxins the condition of the animal may suffer and the failure to produce adequate response may not be due to 'crowding out'.

'Crowding out' of immunity response appears only to occur when one antigen is in considerable excess.

*Non-specific stimulation.* Ramon (1926) has shown that non-specific irritating substances, such as tapioca, increase the antigenic efficiency of 'anatoxine'. Having observed that, whenever the antitoxic value of the blood of a horse increased suddenly, the preceding injections had

resulted in the formation of an abscess, Ramon tested a number of substances and finally decided that tapioca was a most suitable non-specific irritant to add. He prepared 'anatoxine au tapioca' by mixing 100 parts of the antigen with one or two parts of finely powdered tapioca previously sterilized by heating. The injection of this material caused a marked inflammatory reaction, but with aseptic care no abscess resulted. About 80 horses immunized with diphtheria toxoid and tapioca yielded an average value of from 600 to 800 units in comparison with between 375 and 500 units among control horses which had not received tapioca.

Although the antitoxic value of the horses immunized by Ramon with 'anatoxine au tapioca' does not appear particularly high in comparison with the average values obtained at most serum institutions, yet there is a significant difference between these values and those of his control horses. With tetanus immunization even greater differences were obtained. With tapioca, horses averaged 200 to 250 American units (10,000 French units) while the control horses scarcely passed 25 American units after 4 or 5 months immunization. These figures are exceptionally low, and it is necessary to limit the conclusions to the statement that the addition of tapioca will compensate for poor immunization.

Schmidt (1928<sup>2</sup>) used tapioca in diphtheria and tetanus immunization, following Ramon's method. In diphtheria, he obtained a 50 per cent. increase in antitoxic titre as an average, but abscesses occurred and the general condition of the animals was such that tapioca could not be injected continuously. In tetanus immunization, tapioca appeared to be specially active and used with a very poor toxin gave the highest values ever obtained by Schmidt (4,400 International units or 35 German units).

Ramon and Descombey (1927) have shown that the protection of horses against tetanus is more easily obtained by two injections of anatoxin with tapioca or even with olive oil than with anatoxin alone. They show that 1 c.cm. of serum from a horse taken 10 days after the second of the two injections separated by one month, protected against from 1 to 20 guinea-pig fatal doses of toxin if tapioca had not been used, against 50 to 100 fatal doses if killed streptococci had been added to the anatoxin, and against 100 to 1,000 fatal doses if tapioca had been added.

Nattan-Larrier, Ramon and Grasset (1927) injected horses with 10 c.cm. of anatoxin repeated one month later, and showed that 10 days after the second injection of anatoxin alone 1 c.cm. of serum neutralized 5 to 20 guinea-pig m.l.d. After the second injection of anatoxin with 1 gm. of atoxyl or 3 gms. tryparsamide, 1 c.cm. neutralized 50 to 200 fatal doses, and after the injection of anatoxin with tapioca, 100 to 500 fatal doses were neutralized by 1 c.cm.

Other authors have tried similar non-irritating substances. Turpentine is largely used in veterinary medicine as a counter-irritant. Glenny, Pope, Waddington and Wallace (1926<sup>1</sup>) showed that the addition of

0·2 c.cm. of turpentine to diphtheria toxoid injected subcutaneously into guinea-pigs reduced the latent period before antitoxin is formed from 4 weeks to 16 days. They also stated that toluol acts similarly. Glenny and Waddington (1928) gave further evidence of the increase in antigenic response produced by the addition of turpentine or toluol to diphtheria toxoid. They showed that 0·5 c.cm. of this toxoid with the addition of turpentine or toluol was as effective as 10 times that amount without addition. Pico and Ferrari (1925) showed that the formation of an abscess caused an increase in antigenic response even when the irritant was not injected at the same time as toxin. Horses were injected with 0·5 c.cm. of essence of terebenthene 4 days before inoculation with toxin. In 10 horses, the increase in antitoxic titre was 38 per cent. compared with 10 per cent. in the controls.

There is considerable evidence that the injection of a slightly toxic antigen causes a better response than that of an antigen entirely free from toxicity. Park and Zingher (1924), recording their experiments with the use of modified toxins for human immunization, state that 'it is of advantage to have a slight excess of free toxin (from 1/5th to 1/20th minimal lethal dosage in each dose) in the toxoid'.

Glenny, Hopkins and Pope (1924) state that they have considerable evidence that, unit for unit, the antigenic value of a partially modified toxin was better than that of toxin completely modified.

Glenny, Pope, Waddington and Wallace (1925<sup>1</sup>) state that the addition of sublethal doses of diphtheria toxin to completely modified toxin considerably increased the antigenic value as measured by the immunity index. There is some evidence to show that other toxic substances may act in the same way; in a single experiment the antigenic value of diphtheria toxoid was improved by the addition of *B. welchii* toxin. Mason (personal communication) has found that the antigenic value of lamb dysentery toxoid is considerably increased by the addition of a little of the toxin.

It has been clearly demonstrated that the addition of potassium alum to toxoid increases antigenic response. Evidence to this effect is brought forward by Glenny, Pope, Waddington and Wallace (1926<sup>2</sup>) and by Glenny and Waddington (1928). The author has found that the addition of potassium alum to tetanus toxoid is a considerable advantage in the preliminary immunization of horses. Guinea-pigs given 2 subcutaneous injections of 0·1 c.cm. of tetanus toxoid alone after 4 weeks' interval failed to withstand 0·02 test dose (4 m.l.d.) of tetanus toxin, those injected with the same dose of the toxoid to which 0·01 or 0·1 per cent. alum had been added survived 100 test doses (about 20,000 m.l.d.) within 2 weeks of the second injection. It is suggested that different types of toxin may vary, and that one local irritant may improve one type of antigen and not another.

Ramon and Zoeller (1926) have stated that advantage can be taken of the addition of a local irritant by making use of associated vaccines

such as the suspension of a typhoid vaccine in diphtheria toxoid. They have successfully immunized animals by means of a 'vaccin associé' consisting of diphtheria anatoxine and T.A.B. vaccines.

#### METHODS OF TESTING ANTIGENS.

There are two purposes for which antigens are used : the immunization of man and the hyperimmunization of horses to produce antitoxin on a large scale. For the latter purpose, the combining power of a toxin or a toxoid gives a good general indication of its value for immunization, but, as is shown in a later section, there are qualitative differences between different batches of toxin and toxoid, so that the combining value, most easily determined in Lf units, is not sufficient to determine its utility.

An efficient prophylactic for human immunization must be as free as possible from both specific and non-specific factors as well as being an efficient antigen. The presence of free toxin likely to cause severe reactions in the non-immune can be detected with certainty in both guinea-pigs and rabbits, but a non-specific effect cannot be detected. In this section, only the antigenic efficiency and not freedom from non-specific effects will be dealt with.

There are many indications in past work that there are at least two bases of comparison of antigens : the amount of antitoxin produced in an animal as a result of a single injection irrespective of the time taken to reach the maximum concentration in the blood, and also the rapidity with which an animal will produce some antitoxin or even become potentially immune.

The method of testing an antigen should depend upon the use for which it is intended. The following are the available methods of testing antigenic efficiency :

##### *1a. Antitoxin production—primary stimulus.*

The object of injecting an antigen is to stimulate the production of antitoxin so that the injected animal will be able to resist infection. It follows, therefore, that the most direct method of testing is the determination of the antitoxin produced as a result of a given injection. Rabbits or guinea-pigs are most suitable for this purpose. The advantage of using rabbits is that they are more easily bled, and, thus, the course of antitoxin production can be traced in detail. Hartley (1925) has used this method on guinea-pigs to compare the antigenic value of different precipitates formed when toxin and antitoxin are mixed. He bled the guinea-pigs 6 weeks after the injection, and used the results of the titration of this bleeding to give an indication of the tests to be made upon the final sample, taken 11 to 12 weeks after the injection. By this method, the antigenic efficiency of any preparation is determined by the antitoxic value of a number of guinea-pigs three months after the injection of a given dose. With some types of antigen, these readings may not give the maximum response, and they fail to give an indication of the rapidity

with which antitoxin is first produced. By the use of rabbits, however, frequent bleedings can determine the length of the latent period and the time and extent of maximum antitoxic content.

A single injection of a powerful antigen does not produce much antitoxin, rarely more than 1 unit per c.cm., while many antigens, useful for human immunization, produce scarcely any detectable antitoxin as a result of a single injection.

#### *1b. Antitoxin production—secondary stimulus.*

It is sometimes convenient to be able to settle within a short space of time whether there is any antigen left in a certain material. One disadvantage of the primary stimulus method is that traces of antigen will produce no detectable antitoxin and moderate amounts will not produce antitoxin until some 4 to 6 weeks after injection. It has already been shown that secondary stimulus responses can be obtained with a very small quantity of antigen, less than 1/100th of that needed to produce a detectable primary stimulus response, and further, that a secondary response occurs within a few days. Therefore, when it is necessary to determine rapidly whether material is antigenic, injection into an immune rabbit will enable an early determination to be made. This test must, however, be regarded as a qualitative rather than a quantitative test for the presence or absence of antigenic material.

#### *2a. Tolerance—lethal dose.*

The amount of toxin that an immune animal can tolerate depends mainly, if not entirely, upon the antitoxic content of the blood. This test determines whether an animal can survive the injection of a certain dose of toxin at a definite interval of time after the primary injection. Only one test can be made on each animal, and the test cannot be quantitative unless a large number of animals are used. There is no sound evidence of permanent cellular immunity apart from antitoxin. The tolerance of an animal to toxin must be measured in terms of the combining power of the toxin and not in terms of minimal lethal doses. Theobald Smith (1907, 1909) measured the degree of immunity in guinea-pigs born of immunized mothers by injecting the young with a mixture of one unit of antitoxin and various amounts of toxin above the L<sub>+</sub> dose. The difference between the amount of toxin in a mixture that an animal will just tolerate and the amount in a mixture just tolerated by a normal guinea-pig, i.e. 'the rise in L<sub>+</sub> dose', was regarded as a definite function of the antitoxic content of the immune animal. Anderson (1906), in his work on maternal transmission of immunity, established the fact that the young of guinea-pigs used for testing diphtheria toxin and antitoxin could tolerate an L<sub>+</sub> mixture. As a qualitative test, the tolerance for an L<sub>+</sub> mixture is more reliable than the tolerance for one or two minimal fatal doses.

Südmersen and Glenny (1911), working on the production of immunity in guinea-pigs and transmission to young, state that 'the degree of

immunity depending presumably upon the circulating antitoxin is more accurately expressed by the neutralizing value in terms of the volume of toxin (indicating the number of combining units) rather than the number of fatal doses tolerated'.

The Therapeutic Substances Act Regulations, 1927, of the British Government stipulate that diphtheria prophylactic can be accepted as passing the test for immunizing property if 3 out of 5 guinea-pigs (each weighing 250 to 350 gm. and injected with not more than 5 human doses) survive the subcutaneous injection of 5 m.l.d. of diphtheria toxin into each guinea-pig. If this is regarded as a test to show that the material is not devoid of immunizing properties, then the absence of any mention of combining power in the regulations is immaterial, but preparations would pass these tests more easily if a fresh batch of toxin with a high toxin-toxoid ratio were used than with an old toxin with a lower ratio and therefore higher combining power in relation to fatal dose.

### *2b. Tolerance—Schick reaction.*

The Schick reaction in a man is a typical example of a tolerance test for immunity. A fixed dose of toxin is employed in the test; a person with more than a given amount of circulating antitoxin is able to neutralize the toxin injected and no specific reaction occurs; a person with less antitoxin exhibits a positive reaction because some, if not all, the toxin remains unneutralized. The level of immunity generally accepted, as measured by the Schick test, is 1/30th of a unit of antitoxin per c.cm., but all observers do not agree upon the exact figure. This disagreement may be due to three causes: (1) the amount of toxin neutralized by a given amount of antitoxin depends upon the combining power of the toxin and this may vary with different toxins used by different observers who have standardized their Schick dose as 1/50th m.l.d. and not by its combining power for antitoxin; (2) antitoxin titration of the blood of patients may in some instances have been made on samples taken some days after the injection of Schick test toxin, when a secondary response may have occurred in the potentially immune; (3) the amount of the patient's blood available for neutralization of the toxin may vary according to the condition of the injection and even the blood-pressure of the individual; recent work by Glenny and Waddington (1929) appears to show that the toxin injected intradermally is neutralized by the antitoxin content of the tissues in the immediate neighbourhood, but there is usually a state of equilibrium between this tissue antitoxin and that in the circulation.

The Therapeutic Substances Act Regulations, 1927, accept as an alternative tolerance test to the survival of five fatal doses of toxin an intracutaneous test with Schick toxin. Three out of five guinea-pigs injected with a quantity of diphtheria prophylactic not exceeding five human doses must show no reaction after the intracutaneous injection of a Schick dose of toxin given not later than six weeks after the primary injection.

*2c. Tolerance—quantitative Schick test.*

The Schick test may be made quantitative if a series of injections are made with graduated doses of toxin. Glenny, Hopkins and Pope (1924) compared the antigenic value of a batch of concentrated toxoid with that of a batch of unconcentrated toxoid by injecting intradermally multiples of Schick doses of toxin 14 days after the subcutaneous injection of 1 and 5 c.cm. of two preparations. De Potter (1924) passively immunized a number of guinea-pigs and compared the reactions produced by the intracutaneous injection of a series of doses consisting of 1/50th, 1/25th, 1/15th and 1/10th of an m.l.d. of toxin with the antitoxic content of the blood. He found a tolerance to 1/50th of an m.l.d., i.e. one Schick dose, corresponding to an antitoxic content of 1/250th of a unit. For accurate work, the series of doses injected should be determined by combining power and not measured as fractions of the m.l.d. This test may be regarded as the simplest method of fixing the antitoxin level on a single occasion, but cannot be used to trace the course of antitoxin production. Use may be made of the short latent period between a secondary injection and the subsequent rise in antitoxic content by injecting the animal with 3 doses of toxin, e.g. 1/10th, 1- and 10-fold Schick doses, followed 24 hours later by a second series finely graded according to the indications given by the reactions of the initial bracketing series. This method has been applied by Glenny and Waddington (1929), who suggest a further modification, making the ' Quantitative Schick Test ' more generally available. They prepared a series of toxins either fresh, naturally matured or blended with toxoid of such compositions that the specific toxicity of each preparation corresponded to that of the routine Schick dose, but the combining power for antitoxin varied. By the use of such preparations it is possible without danger of undue reaction in animals or man to determine any level of antitoxic content from one-half to two hundred times that determined by the Schick test.

*3a. Potential immunity test—immunity index method.*

This method was originally suggested by Glenny, Allen and Hopkins (1923), who made use of the secondary stimulus effect of Schick doses of toxin to detect the rapidity with which guinea-pigs became sufficiently potentially immune to react to the Schick toxin so that by the following week they would be Schick-negative. The immunity index method must be regarded as the simplest of all methods for giving some quantitative indication of antigenic efficiency. Guinea-pigs are injected with an antigen, and Schick tests are made weekly commencing three weeks after the primary injection. Each injection acts not only as an indicator to show whether the guinea-pig is yet immunized beyond a given level, but also as a secondary stimulus directly the animal has reached a sufficient degree of potential immunity. The immunity index of the antigen is given as 1, 2 or 3, &c., according to whether the first, second or third, &c., Schick dose was the first failing to give a positive reaction. It

is obvious that an antigen with an immunity index of 1 is capable of immunizing a guinea-pig within three weeks beyond the Schick level without the additional help of a secondary stimulus. To such an antigen, the rapid immunity index method is applied.

Details of comparative tests of many types of antigens by this method have been published by Glenny and Waddington (1928).

### *3b. Potential immunity test—rapid immunity index method.*

The routine immunity index method for testing antigenic values cannot show differences between powerful antigens that will render a guinea-pig Schick-negative in three weeks, and the rapid method is adopted. The shortest latent period yet recorded between the injection of a primary stimulus into a guinea-pig and the production of immunity beyond the Schick level is ten days. For this reason, ten days after injection has been adopted as the commencing date for Schick testing in the rapid method. The tests are repeated every two days and the index is given as the number of days after the primary stimulus that elapse before the guinea-pig is Schick-negative, and not the number of injections as in the routine immunity index method. This procedure is adopted to prevent confusion between the two indexes.

### *4. Combined primary and secondary response.*

Antigens suitable for human use have not yet been produced of sufficient potency to immunize successfully by means of a single injection. The power of an antigen, therefore, to induce high immunity in a guinea-pig after a single dose is not necessarily an index of its value for human immunization, but is of use in comparing the relative strengths of different batches of the same type of preparation. The more logical method consists in comparing the immunity response of guinea-pigs injected with a series of doses reduced according to the relative weights of man and the test animal at intervals of time corresponding to those used in human immunization. The degree of immunity reached may be tested by antitoxin production or by the tolerance test. This method may also be used to determine the most advantageous method of spacing doses of a given type of antigen. The author has recently adopted the following procedure as a routine method. Guinea-pigs are injected with 1/100th of a human dose of the prophylactic and the injection repeated four weeks later. Schick testing is commenced ten days after the second injection. By this method of testing, the author has obtained better immunity with the use of toxoid-antitoxin floccules than with toxoid alone, whereas single injections of floccules have never proved so successful as those of toxoid alone.

#### *Comparison between Methods of Testing Antigenic Efficiency.*

It is probable that there is a relationship between the amount of circulating antitoxin in an animal and the amount of toxin that can be injected subcutaneously without causing death, or intracutaneously

without causing a skin reaction. The individual variation existing between guinea-pigs that have been immunized by the same dose of the same antigen makes it difficult to establish this relationship definitely.

Hartley (1925), testing the antigenic efficiency of toxin-antitoxin floccules, injected a number of treated guinea-pigs with 3 m.l.d. of toxin after he had withdrawn blood for antitoxin estimation. An analysis of his figures shows that after the injection of 3 m.l.d. of toxin, guinea-pigs containing 0·005 or less units of antitoxin per c.cm. die within 3 days ; 0·005 to 0·01 within 3½ to 7 days ; those containing 0·01 to 0·02 survive with large local reaction ; and 0·02 or more, with small or no reaction. From these figures it would appear that tolerance to the subcutaneous injection of 3 m.l.d. of the particular toxin used corresponds to an antitoxic content of 0·01 unit per c.cm. In these tests among guinea-pigs with less than 0·01 unit per c.cm., 17 died and 2 survived ; among those with 0·01 unit per c.cm., 2 died and 7 survived (5 with large reactions) ; and among those with more than 0·01 unit per c.cm., all of 18 survived.

Glenny and Waddington (1929) have shown that guinea-pigs just survive 600 fatal doses of toxin given 24 hours after the subcutaneous injection of 100 units of diphtheria antitoxic serum. The toxin used in their experiment contained 20 m.l.d. per Lo dose, so that the subcutaneous injection of 100 units of antitoxin was followed 24 hours later by the tolerance to that quantity of toxin that was equivalent to 30 to 50 units of antitoxin. Twenty-four hours after the subcutaneous injection of antitoxin, nearly half the antitoxin injected appears available to neutralize toxin injected subcutaneously. If the toxin is injected intracutaneously, far less antitoxin is available for neutralizing toxin. About one unit of antitoxin must be injected subcutaneously into guinea-pigs in order that they may tolerate a Schick dose of toxin 24 hours later without a reaction. This amount of toxin is equivalent to 1/1,000th of a unit of antitoxin. We have thus a striking difference between the proportion that is available for neutralization of toxin according to the method of injection.

It appears probable that the amount of toxin that will be tolerated by an animal injected subcutaneously, depends upon the total antitoxic content of the whole body and not upon the concentration of antitoxin in the blood. On the other hand, the amount of toxin that can be injected intracutaneously without causing a reaction appears to depend upon the antitoxic concentration and not the total present. It is possible that a limited amount of circulating antitoxin comes into contact with toxin injected into the skin before that toxin becomes fixed to the tissues, but it is suggested that circulating antitoxin is of less importance than the actual cell-content of antitoxin, and that the amount of antitoxin available to neutralize the injected toxin is that amount contained in the tissues of the limited area entered by the toxin. This amount of antitoxin would depend upon the exact technique of the injection, and for a

given area exposed would bear a fixed relation to the concentration of antitoxin in the blood if a state of equilibrium had been reached.

Glenny and Waddington (1929) have shown that guinea-pigs injected intravenously with diphtheria antitoxin may be Schick-positive when tested within a few hours of injection and later become Schick-negative. They found that sufficient antitoxin must be injected to give 1/25th of a unit of antitoxin in the circulation in order to make a guinea-pig Schick-negative when tested 10 minutes after injection. When equilibrium between tissues and blood has been established 24 hours after passive immunization or in actively immune animals, the Schick level corresponds to between 1/250th and 1/500th of a unit of antitoxin per c.cm. of the blood. This level is slightly lower than that found by Henseval and Clevers (1923<sup>1</sup>) and by de Potter (1924) in guinea-pigs 6 hours after the intravenous injection of antitoxin.

In man the Schick level of immunity has been taken generally as corresponding to 1/30th of a unit of antitoxin per c.cm. of blood ; this level was first determined on a limited number of children by Michiels and Schick (1913) and confirmed by Kolmer and Moshage (1915). Since that date there have not been sufficient experimental data to warrant deviation from these figures, although, as it has already been pointed out, there must be wide deviation according to the technique employed. It appears very probable, however, that the Schick level in man is much lower than that originally supposed. The number of border-line reactions is small and large groups must be tested to establish a definite level. Moloney and Fraser (1929) found 65 Schick-positive reactors all to contain less than 1/50th unit per c.cm. Of the Schick-negative reactors, 11 contained over 1/10th unit, 5 over 1/25th and 1 under 1/50th. It appears probable that the majority of Schick-positive reactors contained less than, say, 1/200th unit, and the majority of Schick-negative over 1/25th unit, but it has not been established what is the proportion of negative and positive reactors of those with an antitoxic content between these values.

#### DIPHTHERIA PROPHYLACTIC.

The essential features of a successful preparation for human immunization are absence of excess of specific or non-specific toxicity combined with high antigenic efficiency. There is little, if any, evidence to show that the presence of horse-serum should be avoided ; but any danger of serum sensitization can be prevented by the use of antitoxin of bovine origin. The efficiency of a preparation as an antigen and its freedom from excess of specific toxicity can be tested on laboratory animals, but non-specific effects can only be judged by results in the human subject. Unaltered toxin is of no value for human immunization because doses that can easily be tolerated by all subjects are too small to have any antigenic value ; toxin is therefore combined with antitoxin or rendered non-toxic by chemical or physical means. The following are the chief preparations that have been extensively used or are suitable for use : toxin-antitoxin

mixtures—3 L+ mixtures or 1/10th L+ mixtures; toxoid-antitoxin mixtures; toxin-antitoxin floccules; toxoid-antitoxin floccules; toxoid, undilute or dilute; sodium ricinoleate toxin or toxoid.

*Toxin-Antitoxin Mixtures—Behring's T.A.*

The use of toxin-antitoxin mixtures for human immunization was first suggested by Smith (1907). The first application of this suggestion was made by v. Behring (1913), but no details of preparation or methods of testing were published. Rohmer and Levy (1921) state that the more toxic of Behring's preparations, TA VI, contains free toxin to the extent of 1,000 Ln (minimal reacting doses or m.r.d.) per c.cm. and the less toxic, TA VII, contains one-tenth of this amount of toxin free. Henseval and Clevers (1923<sup>2</sup>) tested batches of Behring's mixtures and found that doses of 0·5 c.cm. or more of TA VI killed guinea-pigs in 48 hours, 0·25 c.cm. killed in 5 days and 0·1 c.cm. in 9 days. The injection of from 1·0 c.cm. to 5·0 c.cm. of TA VII was followed by death in 3 to 4 days; 0·1 to 0·5 c.cm. caused paralysis after one month. The toxin-antitoxin mixtures of v. Behring are diluted before injection; the degree of dilution is modified in subsequent injections according to the reaction produced by the first injection; the usual procedure is to inject every 2 days 0·1 c.cm. of dilutions of increasing strength until a reaction is produced.

*Toxin-Antitoxin Mixtures—T.A.M. 3 L+.*

The toxin-antitoxin mixture introduced by Park (1913) was less toxic than that of v. Behring. The earlier mixtures were made with undilute toxin slightly over-neutralized with antitoxin. The toxin used contained from 2·5 to 5·0 L+ doses per c.cm. Later neutral mixtures contained 1·125 to 1·25 units of antitoxin to each L+ dose; the injection of 5·0 c.cm. into guinea-pigs did not cause acute death but resulted in late paralysis. Zingher (1917) describes the method of preparation of these mixtures. The toxin used is allowed to stand for at least six months to become stabilized. Toxin containing less than 2·5 L+ doses is not used.

There were two objections to the use of undilute toxin in this mixture: severe non-specific reactions, particularly in adults, and the danger of dissociation causing severe toxic symptoms such as those reported by Kelley (1924). There is a possible further objection in the formation of toxin-antitoxin floccules, so that much of the antigenic material may be removed by filtration.

*Toxin-Antitoxin Mixtures—T.A.M. 1/10 L+.*

Schroder and Park (1923) endeavoured to reduce the non-specific reactions caused by 3 L+ mixtures. They could not find any method of purifying the toxin in order to separate the irritating bacterial substances in the culture fluid from the valuable specific toxin. It had been observed, however, that the immunizing power of toxin-antitoxin mixtures made from stronger toxin (6 L+ doses per c.cm.) was no greater than that of mixtures made with toxin of half that strength so neutralized

as to leave the same amount of free toxin. This suggested that the same antigenic response might be obtained with the use of weaker or dilute toxins. As a result of testing various dilutions it was found that toxin-antitoxin mixtures of good antigenic efficiency could be prepared containing only one-thirtieth of the total amount of toxin present in the original 3 L+ mixture. Toxin diluted to contain 1/10th L+ dose per c.cm. to which 0·75 unit of antitoxin was added to each L+ dose constituted the 1/10th L+ mixture adopted by them. Such mixtures injected into guinea-pigs in doses of 1·0 c.cm. caused paralysis followed by death in about 18 days and 5·0 c.cm. caused death in 10 days. Schroder and Park showed that more toxic mixtures produced higher immunity.

The 1/10th L+ mixture has been extensively used in America and has been found to be an improvement on the former mixtures. There is no recorded instance of increased toxicity occurring after these dilute mixtures have been frozen ; the small amount of toxin originally present greatly lessens, if not entirely prevents, the possibility of serious accidents. There is some evidence to show that dilute mixtures are less stable than the original 3 L+ mixtures, but they are sufficiently stable for practical purposes, and little or no deterioration can be detected in their antigenic efficiency after they have stood for six months at room temperature.

Banzhaf (1924) describes the method of preparation of the 0·1 L+ mixtures used by Park and his colleagues. 'The toxin is prepared from veal broth to which 1 per cent. of glucose has been added. Special precautions are taken to limit bacterial growth during the preparation of the broth ; meat is infused in a refrigerator and next morning the infusion is boiled to destroy most of the organisms. The infusion is, however, fermented with *B. coli*. Toxin is stored for at least one year before use. Trial batches of T.A.M. are made containing 0·75, 0·77 and 0·8 unit of concentrated antitoxin to each L+ dose of toxin. The mixture which causes death in from 5 to 10 days with 5 c.cm. doses in 300 gm. guinea-pigs and paralysis with death with 1 c.cm. doses in 250 to 260 gm. guinea-pigs has the proper amount of antitoxin added to each L+. If the 5 c.cm. dose guinea-pigs die as stated and the 1 c.cm. dose guinea-pigs show no definite symptoms of paralysis the toxin is considered too low in that portion of toxin which causes paralysis and is unsuitable for toxin-antitoxin mixtures'. It may be suggested here that the absence of paralysis may not be an indication of the absence of a particular constituent of the toxin, but may be indicative of firm combination between toxin and antitoxin, and therefore less dissociation, freeing toxin available as an antigenic stimulus. When the constitution of the T.A.M. has been settled the toxin and antitoxin are mixed in correct proportion and then diluted.

#### *Toxoid-Antitoxin Mixtures.*

The use of mixtures containing toxin modified by formaldehyde was first suggested by Glenny and Hopkins (1923<sup>4</sup>). They reported the

successful use in human immunization of a mixture of modified toxin and antitoxin diluted 1 in 10. Glenny, Hopkins and Pope (1924) showed that 'in an immunizing mixture prepared with modified toxin the amount of antitoxin present does not within wide limits affect the antigenic power'.

One of the advantages of the use of modified toxin or of toxoid over that of toxin in immunizing mixtures lies in the ease of preparation; very careful adjustments in the degree of neutralization must be made when toxin is used and there is only a small margin between over- and under-neutralization, resulting in poor antigenic value or severe reaction. A further advantage is that less serum is needed in toxoid than in toxin-antitoxin mixtures. Again the reduction or absence of toxicity before the addition of antitoxin is a safeguard against any possible freeing of toxin by dissociation or preferential destruction.

In the preparation of toxoid-antitoxin mixtures it is not necessary to use toxin so completely modified that there are no traces of specific toxin remaining. The mixture first used for human immunization described by Glenny and Hopkins (1923<sup>4</sup>) was prepared from toxin so modified that only five guinea-pig fatal doses per c.cm. remained; after addition of antitoxin the mixtures were diluted 1 in 10, so that the immunizing dose (1·0 c.cm.) contained only one-half of a guinea-pig fatal dose even if no antitoxin were present.

The immunizing mixtures most extensively used in Great Britain since the commencement of 1924 have been prepared from either completely modified toxin (toxoid) or from toxin so modified that one human dose of the final mixture would not contain one guinea-pig fatal dose if no antitoxin were added. The amount of antitoxin added corresponds to from 20 to 40 per cent. of the total combining power of the toxoid. The mixture is diluted 1 in 10 immediately after the addition of antitoxin. It has been suggested that the presence of the antitoxin may increase the antigenic value of the toxoid; Glenny (1925<sup>1</sup>) suggests, without any supporting evidence, that the addition of antitoxin delays absorption and by slow dissociation of the toxoid causes a steady and more continued stimulation. The addition of antitoxin may be regarded as a concession to British conservatism; any type of toxin-antitoxin mixture was freely tried while toxoid alone found little support.

#### *Toxoid.*

Glenny and Hopkins (1923<sup>4</sup>) first suggested the use of toxoid alone for human immunization. They state 'it may be possible shortly to use toxin so modified that it will be completely non-toxic without the addition of antitoxin. This would constitute a marked advance in the prevention of diphtheria; the presence of horse-serum in a toxin-antitoxin mixture might possibly cause reactions in serum-sensitive subjects, and possibly sensitize others not already sensitive, although no worker has as yet found evidence of sensitization by such small doses of serum in a human subject'. Owing to the disturbing influence of phenol these authors had not by that

date succeeded in reproducing the completely atoxic preparation reported by Glenny and Südmersen (1921), but they demonstrated good immunity in guinea-pigs following the single injection of a fraction of a lethal dose of modified toxin.

Almost at the same time Schroder and Park (1923) immunized children with an old and much deteriorated toxin dilution, of which 1·0 c.cm. caused late paralysis in guinea-pigs. A few months later Ramon (1923<sup>3</sup>) used completely modified toxin upon guinea-pigs and horses and suggested the term 'anatoxine'. This term has not been generally accepted in England or America, where the well-established term 'toxoid' is preferred. Although Ramon was not the first investigator to show that diphtheria toxin could be rendered atoxic by formaldehyde nor the first to show that such atoxic products, always known as toxoid, were antigenic, yet it is to Ramon's flocculation test that we owe much of the recent advance in the preparation of toxoid. Further, Ramon has been more successful in France than workers in other countries in urging the use of such material in human immunization. It is possible that Ramon's 'anatoxine' made from Martin bouillon is freer from non-specific material than toxoids prepared from other types of broth. Park, Banzhaf, Zingher and Schroder (1924) state that undilute toxoid gives non-specific reactions similar to those produced by the 3 or 5 L+ mixtures which had been discarded in favour of 1/10th L+ mixtures.

Glenny, Hopkins and Pope (1924) showed that 0·1 c.cm. of toxoid was equal in immunizing power to 1·0 c.cm. of a 1/10th toxin-antitoxin mixture. They further showed that such toxoid could be concentrated by acetic acid precipitation; when so concentrated it still produces non-specific reaction in man. Glenny, Pope, Waddington and Wallace (1925<sup>2</sup>) showed that 'the addition of sublethal doses of diphtheria toxin to fully modified toxin considerably increased the antigenic value as measured by the immunity index in guinea-pigs'. The specific toxicity can always be determined in guinea-pigs; no danger need therefore arise if modified toxin contains a little toxin in addition to much toxoid.

#### *Toxin-Antitoxin Floccules.*

The precipitate which forms when toxin and antitoxin are mixed in equivalent proportions contains all the specific antigen and antibody with relatively little non-specific material. Experimental evidence of this was communicated to the author in 1923 by Hartley, who only published his results in 1925. Sordelli and Serpa (1925), Schmidt and Scholz (1926), Eberhard (1926) and Glenny, Pope, Waddington and Wallace (1926<sup>2</sup>) have also shown that floccules will immunize laboratory animals. Hartley (1926) showed that the preparation of floccules contained only 1/50th of the nitrogenous material present in the mixture of toxin and antitoxin from which they were made. This reduction of non-specific material would indicate that such floccules will prove to be a particularly useful prophylactic for human use. Extensive comparison between these and

other preparations has not yet been made, but Schmidt and Scholz (1926) and Eberhard (1926) have reported promising results.

Floccules are prepared by adding to toxin the minimum amount of antitoxin that will cause flocculation. The mixture after well shaking is left to stand a week or more in a cool dark place until floccules have settled and the supernatant fluid is clear. The floccules are collected by centrifuging and washing at least six times in saline solution before final suspension.

#### *Toxoid-Antitoxin Floccules.*

Glenny and Pope (1927) showed that floccules formed by the addition of antitoxic serum to toxoid are as good antigens as toxin-antitoxin floccules. They suggest that the use of toxoid in this preparation is desirable in order to avoid all possible danger resulting from dissociation between the toxin and antitoxin that might occur under certain conditions. It appears a sound principle to avoid the use of unmodified toxin in any preparation intended for human use. The history of the various accidents in the past shows that either dissociation has occurred as the result of unforeseen circumstances or mistakes in preparation have been made. The use of toxoid instead of toxin gives a further safeguard.

#### *Sodium Ricinoleate Toxin or Toxoid.*

The action of sodium ricinoleate on toxin and toxoid has been fully discussed on p. 126. Larson and his colleagues have used ricinoleated toxin in human immunization, but even in experienced hands it is a dangerous procedure, because the toxin is only masked and not modified and dilution or precipitation of the soap frees the toxin. The same objection does not apply to the use of toxoid prepared by the action of formaldehyde. Glenny and Pope (1927) immunized guinea-pigs with ricinoleate toxoid, and suggested that such a preparation should replace potentially dangerous ricinoleate toxin. It is possible that sodium ricinoleate masks the non-specific material present in all toxoid preparations and thus the disturbing reactions frequently following the use of undilute toxoid are reduced.

#### *Production of Antitoxin.*

The successful production of antitoxin depends upon three factors : the responsiveness of the horse, the strength and quality of the toxin used, and the size and spacing of the doses given.

##### *CHOICE OF HORSE.*

It has long been accepted that there is large variation in the capacity of different horses for antitoxin production. This capacity depends upon several factors, but chiefly upon the inherent power of antigenic response, which may or may not be the same for different antigens, and the previous history of the animal with regard to subliminal infections with *C. diphtheriae*.

From the general principles of primary and secondary response, it is obvious that much time is saved in immunization if a horse is already potentially immune to diphtheria toxin.

We may divide horses into several classes : (a) Those which, through the absence of all contact with *C. diphtheriae*, have received no primary stimulus and contain no diphtheria antitoxin. Such horses are of necessity slow to immunize, but it is possible that they may eventually produce as high-grade antitoxin as other horses, but much time is lost in preliminary immunization. (b) Horses that have received natural stimulation but not in the immediate past, and whose antitoxic content has fallen below an amount easily detectable, i.e. 1/2,000th to 1/5,000th of a unit per c.cm. Such horses are potentially immune and respond rapidly. Ramon (1928) states that he has obtained antitoxin of a value of 2,200 units per c.cm. in a Schick-positive horse, but does not state whether the horse contained some normal antitoxin below the Schick level. (c) Those horses which, although in relatively frequent contact with infection, have failed to produce antitoxin to any detectable extent owing to their weak capacity for response. Such horses will always fail to produce high-grade antitoxin. (d) Similar horses which through frequent and recent infection still contain considerable amounts of antitoxin but are incapable of extensive response. (e) Those horses which give a naturally good response and have received previous stimulation. Such horses form the best group for the production of antitoxin.

It is obviously important, therefore, to make a preliminary choice of horse on the basis of the amount of normal antitoxin in the serum. Only a limited number of horses in group (b) can be successfully and rapidly immunized. If the supply of horses is sufficiently limited to make it necessary to use every horse likely to be suitable for the purpose, then this second group can be picked out from (a) and (c) by testing their antitoxic content a week after a single dose of diphtheria toxoid ; if the animal is potentially immune, but contains no normal antitoxin, a secondary response will occur in this time.

In the author's experience it is not possible by a single similar test for response to separate horses in groups (d) and (e) ; this can only be done by testing their antitoxic content some two or three weeks after the commencement of intensive immunization.

Various workers test the normal antitoxic content of horses in different ways ; thus, Glenny (1925) states that many years previously he tested the suitability of horses intended for antitoxin production by measuring their antitoxic response to a single injection of toxin. It was found that almost all horses giving a good response had a good antitoxic content, and it seemed unnecessary to test all horses in order to discover the few which possessed potential immunity without antitoxic content. Choice was therefore made according to the natural antitoxic value of the horses, the size of oedema produced by the subcutaneous injection of toxin being used as the measure of their antitoxic content. More recently,

many workers have adopted the Schick test for the horse, but in order to detect a suitable level of antitoxic content stronger test-doses must be used ; thus, Celarek and Porebski (1928) inject 4 or 8 m.l.d. of toxin intracutaneously. Horses showing no reaction contained 1/5th or more unit of antitoxin per c.cm., and subsequent immunization yielded an average of 1,000 units per c.cm. ; those with a positive reaction containing 1/10th of a unit or less averaged 250 units per c.cm.

One disadvantage of the method is that all horses in the stud have thus received injections of diphtheria toxin, lessening their utility for experimental work subsequently. The author therefore prefers to test the antitoxic content of the blood of a horse by means of the ordinary intracutaneous method in guinea-pigs. It is thus possible to know the antitoxic content with reasonable accuracy between 1/2,000th of a unit and some 10 or 20 units, a value occasionally seen in normal horses.

Horses appear to vary in the ease with which they tolerate injections. If toxin and not toxoid is used, injections are naturally graded according to the strength of the toxin and the antitoxic content of the horse, but certain horses appear unable to tolerate injections of either toxin or toxoid without having high temperatures and large swellings. It is possible that some horses which have received early infections with *C. diphtheriae* have developed an allergic condition to diphtheria bacterial protein corresponding to the pseudo-Schick reactors in man.

It is interesting to note that large local reactions seldom follow the injection of tetanus toxoid or of scarlet-fever toxin. No work appears to have been done to determine whether this is due to the nature of the toxin or to the condition of the horse. Does *C. diphtheriae* produce more harmful products of metabolism than *B. tetani*, or can we link up the absence of natural tetanus antitoxin in horses with the absence of any previous antigenic stimulus which might otherwise have produced an allergic condition ?

#### CHOICE OF TOXIN.

The methods of preparing diphtheria toxins have been fully described by the Bacteriological Committee of the Medical Research Council (1923). Since this date there have been few additions to our knowledge of the subject, but the universal adoption of the flocculation test of Ramon should result in further extensive work on this problem. It must be borne in mind that toxin is produced chiefly for use in the immunization of man or the hyperimmunization of horses. Both of these aims demand that the material should be as free as possible from harmful products of metabolism. The strongest toxins we have used have been made from broth prepared by the tryptic digest of horse-muscle ; such toxins unfortunately cause severe reactions in horses and this reduces their utility for immunization.

Culture filtrates of *C. diphtheriae* vary considerably in three respects : (1) the total amount of necessary antigenic material present, i.e. toxin

plus toxoid ; (2) the degree of toxicity in relation to total antigen present ; (3) the presence of harmful material. The strength of specific antigen, i.e. toxin plus toxoid, can be measured by its combining power with antitoxin. In the early days of immunology the strength of toxin was judged by the m.l.d., but it was soon realized that toxoid played an equal part in immunization and this measurement was replaced in many laboratories by the Lo or L+ tests, which formed an easier, quicker and more exact measurement of the antigen. Later advances led to the use of the Lr dose and finally the Lf dose. It was, however, not until this last advance by means of the flocculation reaction of Ramon that the measurement of combining power was universally adopted and the m.l.d. test abandoned by all workers. Even now, strangely enough, the strength of tetanus and other toxins is quoted in literature in terms of m.l.d. without mention of combining power, and most of the modern work on scarlet fever appears to be based upon m.r.d. rather than upon the combining power with an arbitrarily chosen standard of antitoxin.

As a general rule it may be stated that, other things being equal, a strong toxin, i.e. one capable of combining with a large amount of antitoxin, is preferable for the immunization of horses. The toxin usually employed contains between 10 and 20 Lf doses per c.cm., but far weaker toxin has been successfully used ; for example, the formalinized toxin used by the author in 1904 had, before modification, an Lo dose of about 0·3 c.cm. and therefore presumably contained about 4 Lf doses per c.cm. The results of the immunization of horses with this modified toxin quoted by Glenny, Pope and Waddington (1925) show the average value of antitoxin produced to have been about 600 units per c.cm., a value not very much below that obtained in most institutions at the present day where far stronger toxin is used.

The specific toxicity of the culture filtrates used for immunization is of importance when the method of immunization adopted involves injection of large volumes in the earlier stages of immunization. For such a method it is essential to use toxin partially or fully modified into toxoid. If a method of gradually increasing doses is adopted, specific toxicity matters very little, because horses are chosen already possessing some natural antitoxin to protect them against the initial doses, and the subsequent active production of antitoxin more than keeps pace with the increase in amount of toxin injected.

In the present state of our knowledge, it is not possible to say whether there are inherent qualitative differences in the specific antigen. Ramon is convinced that fast flocculating toxins are better than those flocculating slowly, but this does not necessarily imply differences in the pure antigen. Such differences probably exist but they have not been established.

The non-specific qualities of a toxin are of great importance. In the author's experience, toxins made with different types of broth vary greatly in the effects they produce in horses and marked differences are seen in different batches prepared apparently in the same way. It

appears probable that these non-specific differences are due partly to the amount of bacterial protein produced and partly to substances present in the original broth or produced as products of metabolism during the growth of the organism. Individual horses may show great differences in the way in which they react to these harmful products. Theoretically it may be assumed that the toxin containing a large amount of bacterial protein is unfavourable for immunization owing to interference in immunity response, and also to the limitation of dose that can be given if horses are susceptible to such bacterial protein. Again, it seems to be possible to employ for immunization two batches of toxin produced in apparently the same way and approximately of the same strength and rapidity of flocculation, that, when used upon groups of say 10 horses vary little in the local or constitutional reaction produced, and yet one yields consistently high-grade antitoxin and the other low-grade material. It is impossible at present to say whether such differences are due to qualitative differences in the pure antigen, to the presence or absence of other antitoxic material causing interference in immunity response, to the presence of material increasing or delaying the rate of absorption of the toxin, to the possible presence or absence of accessory factors similar in action to the tapioca of Ramon (1926), or to the turpentine, toluol or alum of Glenny, Pope, Waddington and Wallace (1926<sup>1</sup>).

#### COURSE OF IMMUNIZATION.

The scheme of immunization adopted by any worker must be one applicable to the type of toxin or toxoid used and dependent to some extent upon the type of horse. There are indications from the literature that the degree of natural immunity of horses in different countries varies considerably, but there are no authentic figures on the subject. It follows, therefore, that the average commencing dose easily tolerated by the horse may vary in different countries, and, further, that there will be differences in the allergic state of the horse that will also limit the dose that can be injected without large reactions.

The general aim of immunization is to produce the largest amount of the high value antitoxin in the shortest time without causing undue suffering to the horse. Even when deprived of its specific toxicity by modification with formaldehyde, a culture filtrate is still harmful when injected into horses, although no acute toxæmia is produced. It follows, therefore, that there is a definite limitation to the size of dose that can be injected. If we consider results of experiments in guinea-pigs, which appear less susceptible to these additional poisons, we find that the larger the dose injected the more quickly is antitoxin produced and the higher the resulting level. In horses the amount of antigen that can be injected is relatively small in relation to the concentration of the antibody aimed at ; it is necessary, therefore, so to arrange the injections that the responsiveness of the horse increases and the relatively small stimuli available will produce greater effects, but at the same time it is essential

to maintain the general condition of the horse. Glenny, Pope, Waddington and Wallace (1925) showed that better results were obtained in the immunization of tetanus horses if no injections were given for a period of a month or two after the primary stimulus. Without such a period of rest the course of injections might result to a certain degree in lowering the condition of the horse without such compensating immunity response as occurs after the same course of injections given when the horse had attained a higher power of response.

Three main schemes of immunization have been suggested at different times. Dean (1908) commenced with 0·01 c.cm. of toxin and doubled the dose every two or three days; it appears unnecessary to commence with so small a dose because horses with natural antitoxin amounting to 0·01 or more unit per c.cm. will tolerate without disturbance at least 1·0 c.cm. of toxin. Sordelli (1921) suggested a more rapid course, the dose being trebled on each occasion. This more rapid treatment resulted in considerable saving of time, thirty days only elapsing between the first injection and the date of bleeding. The author is of the opinion that Sordelli's method is not applicable to all toxins and horses, and that such rapid increase of dosage may result in considerable suffering to a horse. Ramon (1928) commenced immunization with much larger doses, 20 c.cm. of 'anatoxine', and increased at a slower rate than either Dean or Sordelli.

All experienced workers will agree that horses need individual treatment and, therefore, any scheme of injections such as those given above must be regarded as a general indication of method only. It is probable that the best method to adopt consists in commencing with an injection of 1·0 c.cm. of toxin or toxoid and rapidly increasing the dose daily until a dose is reached which the horse will just tolerate. This dose may be regarded then as the starting-point of a regular course of immunization. If the animal has been tolerating all doses without reaction then the increase could be continued at the threefold rate of Sordelli. Horses giving bigger reactions can be continued more slowly as in Dean's or Ramon's method. The advantage of this suggested method is that it is possible to avoid the severe reactions that would follow an initial injection of a large dose in some horses, without undue delay in reaching higher doses in less susceptible horses.

It is unnecessary to continue increasing dosage to the limit of endurance in order to obtain the maximum yield of antitoxin. The condition of the horse must be followed closely to avoid the injection of unnecessarily large doses and at the same time the increase in antitoxic value must be followed so that the immunization can be terminated as soon as possible. Every unnecessary injection should be avoided. It is advisable to take frequent experimental bleedings after the horse has been injected for about three weeks. By means of the flocculation test antitoxic determinations can be made in a few hours. Consideration is then given to the rate at which the antitoxic value in the horse is increasing. It is

frequently advantageous to end the first immunization at an early stage although the antitoxic value of the horse may not be above 400 to 600 units. By bleeding the horse at this stage and resting about 10 days a rapid course of injections will result in a two- to threefold increase in value. The indications for ceasing injections are either slowing down in response to stimulation or a lowering of the condition of the horse. Continued injection often causes a decrease in antitoxic value.

A horse can stand a withdrawal of 8 to 9 litres of blood per week for several months or a series of three such bleedings within 8 days repeated at monthly intervals for many years. The maximum antitoxic value is reached in 7 to 10 days after the last injection of the first immunization ; there is a tendency for this interval to decrease after subsequent injections. After a series of bleedings is taken from a horse the antitoxic value decreases rapidly without further injection ; it is necessary, therefore, to continue immunization to maintain a reasonable level of antitoxic titre. There are two main ways of doing this. Some workers take one bleeding only from a horse 7 to 10 days after the last injection, and then, either immediately after the bleeding or on the following day, give another injection, followed by a bleeding 7 to 10 days later. This series of events is continued until the antitoxic value of the horse has fallen below a useful level. The other method, which in the author's hands yields higher values, consists of a series of definite courses of immunization each followed by three bleedings taken at intervals of 3 to 4 days. In order to obtain the maximum amount of antitoxin after each such immunization, the first bleeding is taken 3 to 7 days after the last injection so that the middle bleeding should correspond with the point of highest antitoxin concentration. Ten days later a further immunization is commenced consisting of five injections given on alternate days. In this way three series of bleedings can be obtained during every four weeks.

We have used this method successfully in producing tetanus, *B. welchii*, dysentery and scarlet-fever antitoxins. The highest titre of these antitoxins is reached between the second and fourth immunization, and there is little falling away in value for several years, although it may be necessary to increase the intensity of the later courses of immunization. In the production of diphtheria antitoxin, however, it is possible to reach the maximum titre that any one horse is capable of producing at the end of the second immunization. In subsequent immunizations, it is difficult to maintain a high average value, and, therefore, it is of advantage to bleed a horse out at the end of the second or third course. Our general experience shows that toxoid is preferable during the preliminary immunization of the horse, but without question unmodified toxin must be used in all subsequent injections. It appears relatively easy to maintain an average value of 1,000 units per c.cm. for the second immunization of all horses.

Although some horses are refractory to immunization it is rare to find an animal that will not produce 100 units of antitoxin per c.cm. and

very few fail to produce 400 units. Such horses may usually be detected by preliminary antitoxic determinations after two or three weeks immunization. An antitoxic titre of 2,000 units at the end of the second immunization is by no means rare. Sordelli (1921) immunized a horse that reached 3,200, and Archipoff (1928) records a value of 3,500. The highest value the author has encountered was 2,600 (in-vivo titration).

## REFERENCES.

ABEL, R., 1894, *Deuts. med. Wschr.*, **20**, 899, 936.  
 ABT, G., 1928, *Ann. Inst. Pasteur*, **42**, 1336.  
 ABT, G. & LOISEAU, G., 1922, *Ann. Inst. Pasteur*, **36**, 535.  
 ANDERSON, J. F., 1906, *Bull. U.S. Hyg. Lab.*, No. **30**; 1910, *ibid.*, No. **66**, 9.  
 ANDERSON, J. F. & LEONARD, G. F., 1924, *J. Amer. Med. Ass.*, **82**, 1679.  
 ARCHIPOFF, K. S., 1928, *J. Microbiol.*, **7**, 150.  
 ARRHENIUS, S. & MADSEN, TH., 1902, *Festskr. ved. Indvielsen af Statens Serum Instit.*, Copenhagen, Art. 3.  
 BÄCHER, ST., KRAUS, R. & LÖWENSTEIN, E., 1926, *Z. ImmunForsch.*, **45**, 86.  
 BANZHAF, E. J., 1924, *J. Immunol.*, **9**, 459; 1928, *The Newer Knowledge of Bacteriology and Immunology*, Jordan & Falk, Chicago, 1928.  
 BANZHAF, E. J. & GREENWALD, C. K., 1924, *Effect of Freezing as regards Toxicity of Diphtheria Toxin-Antitoxin Mixtures*. *Proc. Amer. Soc. Immunol.*  
 BAYNE-JONES, S., 1924, *J. Immunol.*, **9**, 481; 1928, *The Newer Knowledge of Bacteriology and Immunology*, Jordan & Falk, Chicago, 1928.  
 VON BEHRING, 1913, *Deuts. med. Wschr.*, **39**, 873.  
 BENJAMIN, E. & WITZINGER, O., 1912, *Z. Kinderhkl.*, **3**, 73.  
 BERTHELOT, A. & RAMON, G., 1925, *C.R. Acad. Sci. Paris*, **180**, 340.  
 BORDET, J., 1903, *Ann. Inst. Pasteur*, **17**, 161.  
 BRAZIL, V. & VELLARD, J., 1927, *Brasil Medico*, Rio de Janeiro, **41**, 1311.  
 BROKMAN, H. & SPARROW, H., 1925, *C.R. Soc. Biol. Paris*, **93**, 371.  
 BRONFENBRENNER, J. J. & REICHERT, P., 1926, *J. Exp. Med.*, **44**, 553.  
 BUTTLE, G. A. H. & TREVAN, J. W., 1928, *Brit. J. Exp. Path.*, **9**, 182.  
 CALMETTE, A. & MASSOL, L., 1909, *Ann. Inst. Pasteur*, **23**, 155.  
 CELAREK, J. & PORĘBSKI, W., 1928, *C.R. Soc. Biol. Paris*, **99**, 1017.  
 COBBETT, L., 1899<sup>1</sup>, *Lancet*, Lond., ii, 332; 1899<sup>2</sup>, *Brit. Med. J.*, i, 902.  
 COCA, A. F., RUSSELL, E. F. & BAUGHMAN, W. H., 1921, *J. Immunol.*, **6**, 387.  
 COPEMAN, S. M., 1927, *Proc. R. Soc. Med.*, **20**, 43.  
 CRAW, J. A., 1905, *J. Hyg., Camb.*, **5**, 113.  
 CRUVEILHIER, L., 1904, *Ann. Inst. Pasteur*, **18**, 41.  
 DALLING, T., 1928, *Vet. Rec.*, **8**, 841.  
 DANYSZ, J., 1902, *Ann. Inst. Pasteur*, **16**, 331.  
 DEAN, G., 1908, *The Bacteriology of Diphtheria*, ed. by Nuttall & Graham-Smith, Camb. Univ. Press, 1908, chap. **16**, p. 500.  
 DOLD, H. & WEYRAUCH, W., 1927, *Z. ImmunForsch.*, **51**, 458.  
 DREYER, G. & MADSEN, TH., 1902, *Festskr. ved. Indvielsen af Statens Serum Instit.*, Copenhagen, Art. 5.  
 DREYER, G. & WALKER, E. W. A., 1914, *Lancet*, Lond., i, 1023.  
 DUDLEY, S. F., 1922, *Brit. J. Exp. Path.*, **3**, 204; 1929, *Quart. J. Med.*, **22**, 321.  
 VON DUNGERN, 1904, *Deuts. med. Wschr.*, **30**, 275.  
 EBERHARD, H. A., 1926, *Z. Hyg. InfektKr.*, **105**, 614.  
 EHRLICH, P., 1898, *Deuts. med. Wschr.*, **24**, 597; 1906, *Collected Studies on Immunity* (J. Wiley & Sons, New York).  
 VON EISLER, M. & LÖWENSTEIN, E., 1911, *Zbl. Bakter.*, Abt. I, Orig., **61**, 271.  
 FEIERABEND, B., 1925, *Cas. Lék. Č.*, **31**, 1133; 1926, *Amer. J. Pub. Health*, **16**, 643.  
 FISCHL, R. & VON WUNSCHHEIM, 1895, *Z. Heilk.*, **16**, 429.  
 FRASER, D. T. & WIGHAM, H. E., 1924, *J. Amer. Med. Ass.*, **82**, 1114.  
 GLENNY, A. T., 1913, *J. Hyg., Camb.*, **13**, 63; 1925<sup>1</sup>, *ibid.*, **24**, 301; 1925<sup>2</sup>, *J. Path. Bact.*, **28**, 241, 251.

GLENNY, A. T. & ALLEN, K., 1921, *J. Path. Bact.*, **24**, 61; 1922<sup>1</sup>, *J. Hyg., Camb.*, **21**, 96; 1922<sup>2</sup>, *ibid.*, **21**, 100; 1922<sup>3</sup>, *ibid.*, **21**, 104.

GLENNY, A. T., ALLEN, K. & HOPKINS, B. E., 1923, *Brit. J. Exp. Path.*, **4**, 19.

GLENNY, A. T. & HOPKINS, B. E., 1922, *J. Hyg., Camb.*, **21**, 142; 1923<sup>1</sup>, *ibid.*, **22**, 12; 1923<sup>2</sup>, *ibid.*, **22**, 37; 1923<sup>3</sup>, *ibid.*, **22**, 208; 1923<sup>4</sup>, *Brit. J. Exp. Path.*, **4**, 283.

GLENNY, A. T., HOPKINS, B. E. & POPE, C. G., 1924, *J. Path. Bact.*, **27**, 261.

GLENNY, A. T., HOPKINS, B. E. & WADDINGTON, H., 1925, *J. Path. Bact.*, **28**, 305.

GLENNY, A. T. & OKELL, C. C., 1924, *J. Path. Bact.*, **27**, 187.

GLENNY, A. T. & POPE, C. G., 1925, *J. Path. Bact.*, **28**, 273; 1927, *ibid.*, **30**, 587.

GLENNY, A. T., POPE, C. G. & WADDINGTON, H., 1925, *J. Path. Bact.*, **28**, 279; 1928, *ibid.*, **31**, 133.

GLENNY, A. T., POPE, C. G., WADDINGTON, H. & WALLACE, U., 1925<sup>1</sup>, *J. Path. Bact.*, **28**, 333; 1925<sup>2</sup>, *ibid.*, **28**, 337; 1925<sup>3</sup>, *ibid.*, **28**, 463; 1926<sup>1</sup>, *Chem. & Ind.*, **45**, 415; 1926<sup>2</sup>, *J. Path. Bact.*, **29**, 31; 1926<sup>3</sup>, *ibid.*, **29**, 38.

GLENNY, A. T. & SÜDMERSEN, H. J., 1921, *J. Hyg., Camb.*, **20**, 176.

GLENNY, A. T. & WADDINGTON, H., 1926, *J. Path. Bact.*, **29**, 118; 1928, *ibid.*, **31**, 403; 1929, *ibid.*, **32**, 275.

GLENNY, A. T. & WALLACE, U., 1925, *J. Path. Bact.*, **28**, 317.

GLENNY, A. T. & WALPOLE, G. S., 1915, *Biochem. J.*, **9**, 298.

GOLDSWORTHY, N. E., 1928, *J. Path. Bact.*, **31**, 525.

VON GRÖER, FR., 1923, *Biochem. Z.*, **138**, 13.

VON GRÖER, F. & KASSOWITZ, K., 1915, *Z. ImmunForsch.*, Tl. 1, Orig., **23**, 108; 1919, *ibid.*, Tl. 1, Orig., **28**, 327.

HARTLEY, P., 1925, *Brit. J. Exp. Path.*, **6**, 112; 1926, *ibid.*, **7**, 55.

HEINEMANN, P. G., 1908, *J. Biol. Chem.*, **5**, 27.

HENRY, H., 1922, *J. Path. Bact.*, **25**, 1.

HENSEVAL, M. & CLEVERS, J., 1923<sup>1</sup>, *C.R. Soc. Biol.*, Paris, **88**, 960; 1923<sup>2</sup>, *ibid.*, **88**, 962.

HIRSZFELD, H. & L. & BROKMAN, H., 1924, *J. Immunol.*, **9**, 571.

HISSE, P. H., JR. & ATKINSON, J. P., 1900, *J. Exp. Med.*, **5**, 47.

HOEN, E., TSCHERTKOW, L. & ZIPP, W., 1927, *Z. ImmunForsch.*, **51**, 349.

HOSOYA, S. & MIYATA, S., 1928, *C.R. Soc. Biol.*, Paris, **99**, 1297.

HUNTOON, F. M. & CRAIG, S. H., 1921, *J. Immunol.*, **6**, 235.

JUNGEBLUT, C. W., 1927, *J. Exp. Med.*, **46**, 609.

KALIC, D. Z., 1928, *C.R. Soc. de Biol.*, Paris, **98**, 649.

KARASAWA, M. & SCHICK, B., 1910, *Jahrb. Kinderhuk.*, **72**, 264.

KELLEY, E. R., 1924, *J. Amer. Med. Ass.*, **82**, 567.

KELLOGG, W. H., 1922, *J. Amer. Med. Ass.*, **78**, 1782.

KIRKBRIDE, M. B. & DOW, J. E., 1924, *J. Amer. Med. Ass.*, **82**, 1678.

KLIEWE, H. & WESTHUES, M., 1925, *Münch. med. Wschr.*, **72**, 587.

KOLMER, J. A. & MOSHAGE, E. L., 1915, *Amer. J. Dis. Child.*, **9**, 189.

KOULIKOFF, V. & KOMPANEJEZ, A., 1928, *Zhurnal exp. biol. med.*, **8**, 79.

KOULIKOFF, V. & SMIRNOFF, P., 1927, *Ann. Inst. Pasteur*, **41**, 1166.

LARSON, W. P., EVANS, R. D. & NELSON, E., 1924, *Proc. Soc. Exp. Biol.*, N.Y., **22**, 194.

LARSON, W. P., HALVORSON, H. O., EVANS, R. D. & GREEN, R. G., 1925, *Colloid Symposium Monograph*, Chemical Catalog Company.

LARSON, W. P., HANCOCK, E. W. & EDER, H., 1925, *Proc. Soc. Exp. Biol.*, N.Y., **22**, 552.

LARSON, W. P. & NELSON, E., 1924, *Proc. Soc. Exp. Biol.*, N.Y., **21**, 278.

LEDINGHAM, J. C. G., 1907, *J. Hyg., Camb.*, **7**, 65.

LESNÉ, MARQUEZY, LEMAIRE & MONMIGNANT, 1927, *C.R. Soc. Biol.*, Paris, **98**, 1205.

LEULIER, A., SÉDALLIAN, P. & CLAVEL, J. M. C., 1928, *C.R. Soc. Biol.*, Paris, **99**, 1746.

LEWIS, J. H., 1915, *J. Infect. Dis.*, **17**, 241.

LOCKE, A. & MAIN, E. R., 1926, *J. Infect. Dis.*, **39**, 484.

LOCKE, A., MAIN, E. R. & MILLER, F. A., 1927, *J. Infect. Dis.*, **41**, 32.

LONDON, E. S. & ARISTOVSKY, V. M., 1917, *C.R. Soc. Biol.*, Paris, **80**, 756.

LONGCOPE, W. T. & RACKEMANN, F. M., 1918, *J. Exp. Med.*, **27**, 341.

LÖWENSTEIN, E., 1909, *Z. Hyg. InfektKr.*, **62**, 491.

MACCONKEY, A. T., 1912, *J. Hyg., Camb.*, **12**, 507, 511; 1923, *ibid.*, **22**, 473.

MADSEN, T. & SCHMIDT, S., 1926, *Ann. Inst. Pasteur*, **40**, 300.

MEDICAL RESEARCH COUNCIL, 1923, *Diphtheria: Its Bacteriology, Pathology and Immunology*, London, H.M.S.O., pp. 544.

MICHIELS, J. & SCHICK, B., 1913, *Z. Kinderhik.*, Orig., **5**, 255.

MINETT, F. C., 1922, *J. Comp. Path.*, **35**, 291.

MOLONEY, P. J., FRASER, D. T. & FRASER, C. J., 1929, *Ann. Inst. Pasteur*, **43**, 124.

MOLONEY, P. J. & WELD, C. B., 1925, *J. Path. Bact.*, **28**, 655; 1922-5, *Studies from the Connaught Laboratories*, **2**, 257 and 263.

MONTEIRO, J. L., 1921, *Ann. paulist. Med. Cirurg.* **12**, No. **1**, 6.

MORGENROTH, J. & LEVY, R., 1912, *Z. Hyg. InfektKr.*, **70**, 69.

MUTERMILCH, S. & SALAMON, E., 1929, *C.R. Acad. Sci.*, Paris, **188**, 350.

NATTAN-LARRIER, L., RAMON, G. & GRASSET, E., 1927, *C.R. Soc. Biol.*, Paris, **97**, 541.

NÉLIS, P., 1924, *C.R. Soc. Biol.*, Paris, **91**, 1159; 1925, *ibid.*, **92**, 1114, 1116.

NICOLLE, M., CÉSARI, E. & DEBAINS, E., 1920, *Ann. Inst. Pasteur*, **34**, 596.

O'BRIEN, R. A., 1923, *Ann. Rep. Metrop. Asylums Bd.*, p. 124.

PARISH, H. J. & O'KELL, C. C., 1926, *Brit. J. Exp. Path.*, **7**, 173.

PARK, W. H., 1913, *Amer. J. Obstet.*, N.Y., **68**, 1213; 1921, *Arch. Pediat.*, **38**, 384.

PARK, W. H., BANZHAF, E. J., ZINGHER, A. & SCHRODER, M. C., 1924, *Amer. J. Publ. Hlth.*, **14**, 1047.

PARK, W. H. & ZINGHER, A., 1924, *Amer. J. Dis. Child.*, **28**, 464.

PETTIT, A., 1913, *C.R. Soc. de Biol.*, Paris, **74**, 1198; 1914, *Ann. Inst. Pasteur*, **28**, 663.

PICK, E. P., 1912, *Biochemie der Antigene*, G. Fischer, Jena.

PICO, C.-E. & FERRARI, J., 1925, *C.R. Soc. Biol.*, Paris, **93**, 1132.

POPE, C. G., 1927, *J. Path. Bact.*, **30**, 301.

DE POTTER, F., 1924, *C.R. Soc. Biol.*, Paris, **91**, 898.

PREDTSCHENSKY, S. N. & SCHEVIAKOVAN, O. J., 1926, *J. Microbiol.*, **3**, 149.

RAMON, G., 1922<sup>1</sup>, *C.R. Soc. Biol.*, Paris, **86**, 661; 1922<sup>2</sup>, *ibid.*, **86**, 813; 1923<sup>1</sup>, *ibid.*, **88**, 167; 1923<sup>2</sup>, *ibid.*, **89**, 2; 1923<sup>3</sup>, *C.R. Acad. Sci.*, Paris, **177**, 1338; 1924<sup>1</sup>, *ibid.*, **179**, 422; 1924<sup>2</sup>, *ibid.*, **179**, 485; 1924<sup>3</sup>, *Ann. Inst. Pasteur*, **38**, 1; 1925, *Bull. Soc. cent. Méd. Vét.*, **101**, 227; 1926, *Ann. Inst. Pasteur*, **40**, 1; 1927, *C.R. Soc. Biol.*, Paris, **97**, 538; 1928, *Ann. Inst. Pasteur*, **42**, 959.

RAMON, G. & DESCOMBEY, P., 1927, *Ann. Inst. Pasteur*, **41**, 834.

RAMON, G., NOUREDDINE, O. & ERBER, B., 1928, *C.R. Soc. Biol.*, Paris, **99**, 562.

RAMON, G. & ZOELLER, C., 1926, *C.R. Soc. Biol.*, Paris, **94**, 106.

RATNER, B., JACKSON, H. C. & GRUEHL, H. L., 1927, *J. Immunol.*, **14**, 249.

REITER, H. & SOLDIN, M., 1926, *Deuts. med. Wschr.*, **52**, 1337.

RENAUX, E., 1924, *C.R. Soc. Biol.*, Paris, **90**, 964.

ROBINSON, E. S. & WHITE, B., 1928, *J. Immunol.*, **15**, 381.

ROHMER, P. & LEVY, R., 1921, *Arch. Méd. Enf.*, **24**, 585.

RÖMER, P. H., 1909, *Z. ImmunForsch.*, **3**, 208.

RÖMER, P. H. & SAMES, TH., 1909, *Z. ImmunForsch.*, **3**, 344.

ROSENAU, M. J., 1905, *Bull. U.S. Hyg. Lab.*, **21**, 92 pp.

ROSENAU, M. J. & ANDERSON, J. F., 1908, *Bull. U.S. Hyg. Lab.*, No. **43**, 59 pp.

ROUX, E. & MARTIN, L., 1894, *Ann. Inst. Pasteur*, **8**, 609.

SCHICK, B., 1923, *Boston Med. Surg. J.*, **188**, 255.

SCHICK, B., v. GRÖER, & KASSOWITZ, K., 1924, *Handb. biol. Arb. Meth.*, Abt. **13**, Teil 2, Lfg. **137** (Urban & Schwarzenberg), Wien.

SCHMIDT, H., 1926, *Z. ImmunForsch.*, **48**, 217.

SCHMIDT, H. & SCHOLZ, W., 1925, *Arch. Hyg.*, Berl., **95**, 308; 1926, *ibid.*, **96**, 172, 251.

SCHMIDT, S., 1927, *C.R. Acad. Sci.*, Paris, **185**, 1080; 1928<sup>1</sup>, *Ann. Inst. Pasteur*, **42**, 63; 1928<sup>2</sup>, *Acta path. Microbiol. Scan.*, **5**, 129.

SCHRODER, M. C. & PARK, W. H., 1923, *J. Amer. Med. Ass.*, **81**, 1432.

SEDALLIAN, P. & GAUMONT, J., 1927, *C.R. Soc. Biol.*, Paris, **97**, 93.

SMITH, J. HENDERSON, 1907, *J. Hyg. Camb.*, **7**, 205.

SMITH, THEOBALD, 1907, *J. Med. Res.*, **16**, 359; 1909, *J. Exp. Med.*, **11**, 241.

SORDELLI, A., 1920, *Rev. Inst. Bact.*, **2**, 5; 1921, *Rev. Asoc. méd. argent.*, **34**, 199.

SORDELLI, A. & SERPA, R., 1925, *C.R. Soc. Biol.*, Paris, **92**, 824.

STRUBELL, A., 1910, *Zbl. Bakt.*, Abt. **1**, Orig., **53**, 43.

SÜDMERSEN, H. J. & GLENNY, A. T., 1909, *J. Hyg., Camb.*, **9**, 399; 1911, *ibid.*, **11**, 220.

TREVAN, J. W., 1927, *Proc. Roy. Soc. B.*, **101**, 483.  
VALLÉE & BAZY, L., 1917, *C.R. Acad. Sci.*, Paris, **164**, 1019.  
VINCENT, H., 1907, *C.R. Soc. Biol.*, Paris, **63**, 623, 695; 1928, *C.R. Acad. Sci.*, Paris, **186**, 1175.  
WALBUM, L. E., 1909, *Z. ImmunForsch.*, Tl. I, Orig., **3**, 70.  
WALLACE, U., 1927, *J. Path. Bact.*, **30**, 667.  
WALPOLE, G. S., 1915, *Biochem. J.*, **9**, 284.  
WATSON, A. F. & LANGSTAFF, E., 1926, *Biochem. J.*, **20**, 763.  
WATSON, A. F. & WALLACE, U., 1924<sup>1</sup>, *J. Path. Bact.*, **27**, 271; 1924<sup>2</sup>, *ibid.*, **27**, 289.  
WHITE, B. & ROBINSON, E., 1924, *J. Amer. Med. Ass.*, **82**, 1675.  
WILLSTÄTTER, R., 1927, *J. Chem. Soc.*, p. 1359.  
ZINGHER, A., 1917, *J. Infect. Dis.*, **21**, 493; 1921, *Arch. Pediat.*, **38**, 336; 1922, *J. Amer. Med. Ass.*, **78**, 1945.  
ZOELLER, C., 1925, *C.R. Soc. Biol.*, Paris, **93**, 615.

## CHAPTER V. THE PHYSIOLOGICAL ACTION OF SOME TOXINS.

By J. W. TREVAN

(WELLCOME PHYSIOLOGICAL RESEARCH LABORATORIES, BECKENHAM.)

ALTHOUGH much information has been accumulated on the mechanism whereby animals are protected by antisera against the action of toxins, little progress has been made in the analysis of the mechanism whereby the toxins produce their lethal effects. The following account must therefore of necessity be chiefly an enumeration of some of the chasms in our knowledge which need filling, and can only be of value in indicating lines along which it might be profitable to work. The more obvious promise of practical therapeutic results from the investigation of the reactions between sera and toxins is one reason for this state of affairs, but there are others.

One of these is the long latent period of action of many toxins. Glenny (1925) showed that no matter how many lethal doses of diphtheria toxin are injected into a guinea-pig the time of survival after the injection is never less than about 10 hours ; the curve best representing the relation between time of survival and dose is asymptotic to that ordinate at 10 hours. The physiological analysis of the action of diphtheria toxin is therefore a matter of some difficulty because the ordinary physiological experiment is essentially an 'acute' experiment and the maintenance of an animal for as long a period as 10 hours in a relatively normal state under the operative procedures necessary for the analysis of effect on the blood circulation is not easy. In consequence considerably more information has accumulated on the acute action of two bacterial toxins, *B. welchii* and *V. septique*, which do not show a long latent period, than on the action of those that do.

*V. septique* toxin is the easiest to work with. Straub (1919), working with 'Gasödem' toxin, which was probably largely or entirely *V. septique* toxin, was the first to publish an account of the effects of this group on the circulation. It was shown by Dale (note to paper by Robertson, 1919) that this toxin caused in the anæsthetized rabbit alteration in blood-pressure, which could be prevented by the simultaneous administration of antitoxin in adequate amounts. There are four stages to this effect : A preliminary rise followed by a fall, a further rise and a final slow fall. It is suggested by Dale (*loc. cit.*) that this is the result of two opposing factors—a vasoconstriction and a cardiac depression ending in fibrillation. The cardiac action persists after section of the vagi and can be demonstrated on isolated cardiac muscle. The contractions of a rabbit's

isolated auricles suspended in Ringer's solution to which is added the toxin gradually become less frequent and weaker and finally cease. Occasionally an increase of rate is seen at first and rarely an increase in height of contraction. After the auricle has ceased to beat, the addition of adrenaline to the bath will start it again if the dose of toxin has not been too great. Straub (1919) and Lautenschläger (1919) showed that the isolated frog ventricle stopped in systole, and suggested that the action was similar to that of drugs of the digitalis group. The similarity is not complete, as shown by Buttle and Trevan (1928), who only obtained in one experiment out of twenty the initial increase in size of the beat of the isolated auricle, which is characteristic and invariable with drugs of the digitalis group. The action on isolated cardiac muscle can be completely prevented by the addition of antiserum. In the intact animal the ventricle stops before the auricle. The rise in blood-pressure, judging from the action of the toxin on other smooth muscle, is probably due to the direct action on the muscular coat of the arterioles, but has not been more closely studied.

The action on the smooth muscle of the rabbit's uterus has been worked out in some detail (Buttle and Trevan, loc. cit.). *V. septique* toxin prepared by saturation of filtered broth cultures with ammonium sulphate was used. Addition of 2 mgm. to the bath of 15 c.cm. of Ringer's solution produced a contraction. The effects are best seen by adding the toxin after the isolated muscle has started rhythmic contraction in Ringer's solution. There is a suppression of the normal rhythmic contraction and a toxic contraction which passes off after ten to fifteen minutes. When larger doses are used (120 mgm.) the contraction passes off rather more quickly than when the small doses are used, the rhythmic contractions disappear almost entirely, and after about half an hour the muscle becomes irresponsible to adrenaline. With smaller doses (2 to 10 mgm.) partial recovery of spontaneous rhythm takes place. The response to adrenaline after the toxic contraction has ceased varies inversely with the dose of toxin.

There is a latent period which varies inversely with some fractional power, approximately the square root of the dose. Accurate measurement of the latent period is somewhat difficult, but with 1.9 mgm. of a particular toxin no action at all is visible in ten minutes and the contraction reaches its height in just over fifteen minutes, whereas with 30 mgm. the toxic contraction is already decreasing by that time. Adrenaline has a similar but much shorter latent period, which decreases with increasing dose in much the same way. The difference in latent period is presumably due to the more complex molecular constitution of the toxin. The effect is reversible to some extent by washing out with Ringer's solution. The toxin can apparently be removed from the muscle and is not fixed firmly thereto. If, however, the toxin remains too long in the bath, then the effect cannot be reversed by washing, and the tissue dies. The concentration which produces an obvious effect on the uterus in Ringer's

solution is of the same order as that in the serum when a fatal dose is injected intravenously. Thus, taking 44 c.cm. per kgm. as the amount of serum for a rabbit, a dose of 0·027 mgm. per c.cm. of serum injected intravenously failed to kill, and a dose of 0·057 mgm. per c.cm. killed overnight, whereas a dose of 0·03 mgm. per c.cm. failed to produce a contraction of the uterus *in vitro* and 0·06 mgm. per c.cm. produced a definite contraction with a latent period of twenty minutes. The concentration in the serum causing delayed (overnight) death is therefore of the same order as that which produces an effect on smooth muscle in the bath. A concentration of 0·11 mgm. per c.cm. of serum killed in 35 to 40 minutes, whereas the same concentration in the bath caused a contraction in 14 minutes.

The cause of death is almost certainly cardiac failure, at any rate with large doses.

*Desensitization.* Repetition of the toxin dose results under certain conditions in a loss of the sensitivity of the uterus to the toxin so that supramaximal doses are without effect. The desensitization can only be effected when the bath is aerated with oxygen. If a just maximal dose is added to the bath a contraction occurs which disappears in the course of half an hour or so, the normal rhythm of the uterus returning. A dose twice as large can then be added to the bath without effect, and if slowly increasing doses are added at half-hourly intervals as much as ten times the maximal dose will produce no action. The action suggests an analogy with immunity in the whole animal, but it is not specific, for the tissue desensitized to *V. septique* toxin is also insensitive to *B. welchii* toxin. Not only so, but if the process were of the same kind as true immunity, it would be expected that the uterus of rabbit immunized by intravenous injections of the toxin would be insensitive to the toxin ; it is, in fact, not demonstrably different in sensitivity from the uterus of a normal animal. A somewhat similar reaction has been noticed with certain Australian snake-venoms (Kellaway, 1929).

*Neutralization of action by antitoxin.* If an excess of toxin is mixed with a sufficient amount of serum and the mixture added to the bath containing a strip of uterus no contraction is produced. As the amount of serum is diminished, first a small contraction and then larger contractions are produced. Using a dose of toxin forty times the smallest dose causing a contraction, Buttle and Trevan found that the action of 1 mgm. was completely obliterated by 0·00028 c.cm. of a certain serum, and gave a very small contraction in the presence of 0·00022 c.cm. of serum. When mixtures of toxin and antitoxin were injected into mice about 50 per cent. survived when the mixtures contained toxin and serum in the proportion of 1 mgm. toxin to 0·00025 c.cm. of serum.

The amount of serum which produced protection *in vitro* is in this case almost exactly equivalent to the amount which protects the average mouse from death, and this relationship holds for several sera examined. It seems probable, therefore, that the substance in the toxin causing

death of mice is the same as that which produces contraction of the smooth muscle of the uterus. The protection is completely specific. Normal serum of the rabbit does not protect even when it replaces the whole of the Ringer's solution in the bath, nor do other antisera. If the contractions produced by mixtures containing various amounts of excess toxin are compared with the contraction produced by various doses of toxin alone a rough estimate of the amount of 'excess toxin' can be made. Such comparisons show that as serum is progressively added to toxin the first addition of serum 'absorbs' more toxin than the later additions; as the neutral point is approached a smaller fraction of the total toxin is absorbed for each unit volume of serum added. Such a relationship is parallel to that observed with diphtheria toxin (Glenny, Pope and Waddington, 1925).

The combination of toxin and antitoxin is apparently immediate. The amount of antitoxin necessary for neutralization is the same whether the serum and toxin are added separately but simultaneously to the bath or allowed to stand for some hours. If the antitoxin is added after the toxin a larger amount is necessary to neutralize. One minute after the addition of the toxin neutralization can only be obtained by adding two to three times as much antitoxin as is necessary when they are added simultaneously, and this is in spite of the fact that there is no visible change in the contraction of the uterus in that period;  $7\frac{1}{2}$  minutes after the addition of the toxin as much as fifty times the neutralizing dose is required. The contraction will have already appeared when the antitoxin is added but it then disappears and gives place to the normal rhythm. This phenomenon is analogous to what is already known for diphtheria toxin (cf. Glenny and Hopkins, 1925). It is attributed to the toxin becoming gradually more firmly bound to the tissues. But at the stage where a very large excess of serum is needed to neutralize the toxin the latter can be washed out of the uterus by merely changing the Ringer's solution in the bath, and it therefore seems more probable that under these circumstances the increase in the amount of serum necessary is correlated with some difficulty of access of the antitoxin to some part of the muscular structure which contains the active toxin.

The toxin of *B. welchii* produced similar changes in involuntary muscle. This toxin is less easy to work with, as it is more susceptible to destruction by surface coagulation on the bubbles produced by aeration of the bath. *V. septique* shows this phenomenon also, but to a less extent, and for results of a quantitative nature it is necessary to control carefully the rate of bubbling.

*Diphtheria toxin.* The main action of diphtheria toxin appears to be on the circulation. Yabe (1922) has examined the action of the toxin on cats. The cats used were given a large dose of toxin and 18 to 24 hours later they were etherized and decerebrated through the midbrain with Sherrington's apparatus. Such animals had a lower blood-pressure than normal decerebrate animals. The heart, as judged by the rise in

blood-pressure on applying a clamp to the aorta below the renal arteries, was unaffected. Stimulation of the splanchnic nerve caused a normal rise in blood-pressure, but asphyxia caused no rise. Yabe therefore attributes the circulatory failure to a paralysis of the vasomotor centre. From the local vasodilator effect in the Schick reaction it seems likely that the toxin also has a paralytic effect on the wall of the capillary. Buttle (personal communication) has, however, been unable to detect any rise in haemoglobin content in the blood of rabbits dying from the action of other capillary poisons.

The frequent occurrence of haemorrhages into the adrenals has suggested that the circulatory failure is due to deficient secretion of adrenaline. In a guinea-pig *in extremis* from diphtheria poisoning adrenaline is absent from the adrenals, but Buttle (personal communication) has found the same condition in guinea-pigs dying from a variety of other infective processes. Belding and Wyman have shown, however, that diphtheria toxin disappears less rapidly from the serum of adrenalectomized rats than from that of normal rats.

No action of the toxin, even of large doses of concentrated toxin, on smooth muscle or cardiac muscle can be demonstrated *in vitro*, while the auricles of a rabbit just dead from diphtheria toxin revive and beat normally in oxygenated Ringer's solution (Buttle, personal communication).

*Streptococcal toxin.* No analysis of the action of streptococcal toxin has been published. Superficially the action seems to be on the circulation. A large number of attempts to demonstrate an action on isolated tissues in Ringer's solution have given uniformly negative results (Buttle).

*Staphylococcal toxin.* A note in the report for 1929 of the Walter and Eliza Hall Institute, Melbourne, gives a brief account of work with the toxin of the staphylococcus which caused the Bundaberg disaster. The action is to produce circulatory collapse, the main feature of which is a rise in pulmonary blood-pressure. It will be of great interest to see the full account of these experiments.

#### REFERENCES.

- BELDING, D. L. & WYMAN, L. C., 1926, *Amer. J. Physiol.*, **78**, 50.
- BUTTLE, G. A. H. & TREVAN, J. W., 1928, *Brit. J. Exp. Path.*, **9**, 184.
- GLENNY, A. T., 1925, *J. Path. Bact.*, **28**, 251.
- GLENNY, A. T. & HOPKINS, B. E., 1925, *J. Path. Bact.*, **28**, 261.
- GLENNY, A. T., POPE, C. G. & WADDINGTON, H., 1925, *J. Path. Bact.*, **28**, 279.
- KELLAWAY, C. H., 1929, *Brit. J. Exp. Path.*, **10**, 281.
- LAUTENSCHLÄGER, L., 1919, *Arch. exp. Path. Pharmak.*, **85**, 1.
- STRAUB, W., 1919, *Münch. med. Wschr.*, **66**, 89.
- YABE, S., 1922, *J. Pharmacol.*, **19**, 1.

## **CHAPTER VI. GENERAL PROPERTIES OF ANTIGENS AND ANTIBODIES.**

**BY C. H. BROWNING (UNIVERSITY OF GLASGOW);  
WITH A SECTION BY R. MUIR (UNIVERSITY OF GLASGOW).**

### **Introduction.**

**BY R. MUIR.**

IN this and the following chapters we have to deal with the changes in the tissues and fluids of the body which underlie or are associated with the state of acquired immunity. These changes are studied by biochemical, biophysical and histological methods, and the nature and significance of each have to be considered. An account of the development of our knowledge in this domain is given in Volume I, Chapter I, but it will be convenient to recapitulate some of the chief facts. Jenner's method of vaccination against the virus of smallpox and Pasteur's methods of preventive inoculation against chicken cholera, anthrax, &c., all depended on the same principle, namely the introduction into the body of the infective agent in an attenuated yet living condition. Clearly some important change was thereby brought about in the animal treated, but of its nature nothing was then known. The next advance was made by the demonstration by Salmon and Smith in 1886 in the case of hog cholera that immunity could be produced by the injection of the bacilli in the dead state. The importance of this lies in the fact that it showed that the interaction of the living organisms with the cells of the body was not essential for the acquisition of immunity, but that the latter could result from the introduction of the organic material constituting the bodies of the specific organisms. By this time Metchnikoff was carrying out his observations on phagocytosis which demonstrated the all-important difference in the behaviour of leucocytes and other cells in the susceptible and the immune states—the absence of phagocytosis in the former and its presence in the latter. Contemporaneously, researches were being carried out with regard to the bactericidal action of the blood and cell-free serum, and with regard to the means of defence supplied in this way. But while it had been shown that in certain instances immunity, both natural and acquired, might be associated with special bactericidal action of the blood, little progress had been made towards a general explanation of the facts of immunity.

Such is a brief summary of the state which immunology had reached when the next, and in some respects the most important, discovery was made, namely that of the formation of antibodies in the process of immunization. The demonstration by Roux and Yersin in 1888 that a bacterium-free filtrate of a culture of the diphtheria bacillus contained the exotoxin of this organism proved to be fundamental in this connection. It was soon afterwards shown that immunity can be developed against such a toxin, that this is due to the development of a specific neutralizing substance or antitoxin in the blood of the immune animal, and that by means of such antitoxins immunity can be transferred to another animal—that is, that immunity of passive nature can be conferred. This result was first recorded in the case of tetanus antitoxin by Behring and Kitasato in 1890, and, almost immediately afterwards, in the case of diphtheria antitoxin by Behring. It was shown further that antitoxins could be developed against toxins other than those of bacterial origin, for example against the vegetable toxins, ricin and abrin, by Ehrlich (1891). A little later, antitoxin to snake-venom was discovered almost simultaneously by Calmette and by Physalix and Bertrand (1894).

The next advance was the discovery of other properties of antibodies. Pfeiffer in 1894 established the striking fact that in an animal immunized against the cholera vibrio there appear in blood specific antibodies which lead to the death and dissolution of the organisms, and that by means of the serum of such an animal immunity can be transferred to a fresh animal. The main features of antitoxic sera were thus shown to be present in antibacterial sera also. Further, starting from the work of Denys and Leclef in 1895, followed by that of Wright and Douglas in 1903, investigations showed that another property might be possessed by antibodies, namely that of leading to phagocytosis of the homologous organism. Thus the phenomenon of increased phagocytosis previously recognized to be characteristic of the immune animal was found to be due to antibodies. The important facts thus reached are that in active immunity against bacteria antibodies appear in the blood which may act in a defensive manner in two ways, viz. either by leading to the extracellular death of the invading organism or to its phagocytosis and subsequent destruction.

Two other phenomena brought about by antibacterial sera came to be recognized, viz. agglutination and precipitation. These, though not related in the same way to the defence of the body as those just described, are likewise due to the development of antibodies. While at a relatively early date (1889) Charrin and Roger had found that when the *B. pyocyaneus* was grown in the presence of the serum of an animal injected with that organism, its growth took place in small lumps instead of diffusely, it is to Gruber and Durham that we owe the first systematic study of the development of agglutinins in immunized animals, the first paper being published by the latter in 1896. The application of the agglutination test to the diagnosis of typhoid fever was made by Widal

a few months later. In the following year (1897) Kraus showed that on injecting clear filtrates of bacterial cultures into animals their sera acquired the property of forming a precipitate when added to the filtrate used for injection. A precipitating type of antibody or precipitin thus came to be recognized, and subsequent investigation showed that precipitins can be developed in response to the injection of proteins of great variety, an essential condition, however, being that the protein should be foreign to the animal into which it is injected.

It is thus seen that the acquisition of immunity is usually accompanied by the appearance in the blood of antibodies which exert an antagonistic action either against the bacterial toxin or against the vital activity of the bacterium, or which give some physico-chemical reaction with the latter. Further, the faculty of stimulating antibody formation is not peculiar to bacteria and their toxins, but occurs when any of a large number of organic substances are introduced into the tissues, provided that they are of nature foreign to the animal injected. Immunity is now recognized to depend partly on the presence of the antibodies *per se* and partly on accompanying cellular changes, some of which are intimately connected with antibody formation itself. Since the discovery of antibodies a large proportion of the literature has been devoted to a study of their properties and of the substances which give rise to them, the latter being known as antigens. While much has been established in this field of research it must be recognized that practically nothing is known with regard to the constitution of antibodies and the exact nature of their specific action, and while they are usually regarded as proteins modified in some way, even this cannot be stated with certainty.

Another fact of importance is that in the development of acquired immunity we see mainly an intensification of actions and properties possessed by the body in the natural state ; we cannot say that any new method of protection appears. In the natural immunity of an animal various substances either free in the blood or contained within leucocytes and other cells are concerned. These substances may be regarded as being of two classes. One class comprises what may be called normal antibodies, and increase of them occurs during immunization. In the other class are included substances or properties which do not undergo such increase ; of this, complement or alexin is the outstanding example.

In the following chapters there will be given a general account of antigens and antibodies followed by a more detailed consideration of them from the physico-chemical point of view. The various properties of the sera of animals immunized against bacteria or against foreign cells or proteins will then be considered in detail. The properties of complement are treated in Chapter X. Antitoxins are dealt with in Chapters IV and VII.

**Antigens and Antibodies.**

By C. H. BROWNING.

An *antigen* is a substance which on introduction into the tissues of an animal gives rise to the development of an antibody. Between the antibody thus developed and the antigen there is a specific combining relationship, which can be demonstrated by *in-vitro* reactions or by means of animal experiments—for example, by the antibody giving protection against a toxin or bacterium or by its leading to a state of supersensitivity as is seen in anaphylaxis. The nature of antigens will be discussed later in detail, but it may be stated at this stage that the antigen may be in solution, e.g. a toxin or protein, or it may be organized and particulate, e.g. a bacterium or tissue cell. The distinction, however, is not fundamental.

In this chapter the term will be used in the way indicated—as developer of an antibody. The term is also commonly used to denote substances which react with an antibody *in vitro*, although they are by themselves incapable of leading to the development of the antibody *in vivo*. To such substances present in certain alcoholic tissue extracts which react with antibody developed by injections of suspensions of the corresponding fresh tissues, Landsteiner's name of 'haptens' may be applied. In the Wassermann reaction also the conditions appear to be analogous, syphilitic serum reacting with a lipoidal mixture.

In order that any substance should function as an antigen it is essential that it should enter the blood or tissue fluids in an unchanged state. Most antigens when introduced by the alimentary tract have their constitution so altered that they lose their antigenic function. In a few instances, however, the antigen is absorbed unchanged or little changed, and thus gives rise to an antibody. The best known example of this is afforded by the vegetable toxins, ricin and abrin, which Ehrlich showed led to the formation of antiricin and antiabrin on being administered to animals by the mouth. But as a general rule the antigen must be introduced by some other channel than the alimentary canal, that is parenterally. Of the parenteral routes, the subcutaneous, intravenous, intramuscular and intraperitoneal are those generally employed. The most suitable method varies in different cases, and details are given in connection with the various organisms. Different species of animals vary in the readiness with which they respond to particular antigens and in the amount of antibodies which they yield, and variations are also met with in respect to different antigens. Further, even amongst individual animals of the same species variations are met with, as is well illustrated in the production of serum antibodies (precipitins). For example, among a series of rabbits injected with the serum-protein, active antibody-formation may occur in only a small proportion.

*Antibodies* can only be defined as substances which are developed in the living body in response to the presence of antigens. From the historical

survey which has been given, it will be seen that they are recognizable by various phenomena which result from their interaction with their corresponding antigens. In accordance with this, the general practice is to speak of different kinds of antibodies—agglutinins, precipitins, immune-bodies, &c. This practice is convenient and will be followed in the account to be given, but it carries with it the risk of an assumption, namely, that the antibodies designated by such terms are always distinct. The question as to unity or plurality will be considered later in connection with the several accounts under review, but it is important at this stage to recognize that the classification of antibodies is based on their properties, and that each property is recognized only by a particular test. The possibility that the same antibody may give two different recognizable reactions cannot at once be excluded, and there is increasing evidence that this is the case.

We may now refer to some general facts bearing on this subject. The *antitoxins*, the antibodies first to be discovered, act as such by simply combining with and thus neutralizing the toxin, and the union as in some other instances has been shown to be of reversible nature, both toxin and antitoxin being recoverable. Yet the flocculation resulting from their interaction, which has come to be recognized in recent years, acts as a link between the process of simple neutralization and the phenomenon of precipitation. It is amongst the antibodies developed by the injection of bacteria that varieties have been specially distinguished. Two classes of such antibodies are generally recognized—(a) a class in which the interaction with antigen in saline is demonstrable by the use of antibody alone, e.g. agglutinin, and (b) a class in which the presence of a labile substance in the serum called complement is necessary to complete the reaction, e.g. bacteriolysin. Reaction between antigen and antibody may lead to precipitation if the antigen is in colloidal solution (e.g. a serum-protein) or to agglutination if it is particulate; or again it may lead to the phagocytosis of the antigen if it is of organized (e.g. bacterial) or particulate nature. Thus we speak of antibodies corresponding to these results as *precipitins*, *agglutinins* and *bacteriolytins* or *immune-opsonins* respectively. The distinction between agglutination and precipitation, however, depends on the use of antigens in two different states—particulate and soluble; and as will be shown later, the two phenomena are intimately related as regards the physico-chemical changes concerned, and probably as regards their essential nature. Antibodies which require complement for their demonstration are conveniently designated by the generic name of *immune-bodies*, but are often spoken of as *amboceptors* (the name applied by Ehrlich). The essential fact here is that complement unites with the complex antigen *plus* antibody, and the presence of both is necessary for this union. The combination of complement through the medium of immune-body may lead to lysis of the bacteria, erythrocytes, &c.; thus, we speak of a *bacteriolytic* and a *haemolytic immune-body* respectively. In other cases a similar combination of complement with the complex may take place

without lysis of the antigen, the fixation of complement being shown by the disappearance of complement from the fluid when a suitable test is applied. It will be seen that with such a test the presence of antigen can be detected when the corresponding antibody is used, and the presence of antibody when the antigen is used. In this case the term *complement-fixing immune-body* is conveniently applied. Furthermore, it has been found that the opsonic action of a serum leading to phagocytosis may be due to the co-operation of immune-body and complement ; thus we may speak of an *opsonic immune-body*. These terms are convenient merely as indicating the property of any immune-body in question ; whether lytic, complement-fixing and opsonic immune-bodies are distinct or not will be afterwards considered.

Although the immune-bodies first recognized and studied were those developed in response to the injection of antigens of formed nature, such as bacteria, red corpuscles, &c., it was shown by Gengou (1902) that immune-bodies (*substances sensibilisatrices*) could be developed also against colloids in solution, such as blood-serum, milk, &c. In such cases the nature of the antibody can be demonstrated by the fixation of complement which results from the union of antigen and antibody. It may be stated now as a general law that the parenteral injection of a foreign protein gives rise to an immune-body, and accordingly the fixation of complement becomes a delicate test for the antigen or the antibody as the case may be. In many such instances the union of antigen and antibody leads to the formation of a precipitate, and thus there is an important relationship between the physical change of precipitation and the fixation of complement (Chapter X).

Another point of importance with regard to antibody production in general is that an 'antigen' is not homogeneous so far as its antigenic function is concerned, but consists of components which exhibit slight variations in that part of their structure which stimulates antibody formation ; so also the related antibody is not homogeneous, but shows slight variations in its constituents corresponding with those of the antigen. This conception is of fundamental importance in the various serum reactions to be described. Amongst the antigenic molecules of a particular bacterium there may be some peculiar to the species but there are others possessed by allied species. Thus the action of a typhoid antiserum is greatest against the typhoid bacillus, but it also exerts slighter effect against other organisms of the typhoid-coli group, and a precipitin to human serum interacts also in less degree with the serum of anthropoid apes.

With advances of knowledge in this department the old controversy and supposed antagonism with regard to the cellular and humoral theories of immunity has ceased to exist. It has to be recognized, on the one hand, that a state of immunity may be associated with and due to the presence of antagonistic or protective substances in the serum, and that even the movements of leucocytes may be affected by such substances.

On the other hand, the source of all such antibodies is the cells of the body, and we know also that the cells which have been concerned in the production of antibodies undergo change of reactivity, so that they respond differently from normal cells when antigen is again introduced. Further, in artificial local immunity there are phenomena which do not depend upon antibodies but in which an alteration in the cells is the all-important factor. It is also to be noted that the production of antibodies is not related merely to defences of the body but is part of the general law of reaction which occurs when certain foreign molecules are introduced into the tissues or blood. Serology and immunity may thus be regarded as two fields which overlap only in part.

#### *Conditions of Antigenic Action.*

As has been stated, an antigen in the strict sense is the developer of an antibody. Since the latter is a product of the reaction of a living animal, usually a higher vertebrate (see Dungern, 1903)—although cold-blooded animals may react similarly (Schwarzmann, 1927; Ebert, 1929)—it is not surprising that antigenic action varies widely with the species of animal used to test antibody production, and even with different individuals of the same species. It is almost universally true that an animal will not react to antigens derived from its own tissues, i.e. auto-antibody production does not occur, and also that little reaction takes place to antigens originating from other individuals of the same species, i.e. formation of iso-antibodies is usually not marked, although exceptions to both these statements are met with. Thus in the serum of persons who suffer from paroxysmal hæmoglobinuria *e frigore* an autohæmolysin is found (Donath and Landsteiner 1904, 1925) which is formed apparently only in association with syphilitic infection (see Browning and Watson, 1913). Also isolysins can be developed after injections of red blood corpuscles from other members of the same species (Chapter IX). It is the general rule, however, that an antigen is a substance foreign to the tissues of the reacting animal.

As regards the nature of antigens, they are all of undefined chemical constitution and are for the most part closely associated with proteins. Attempts to demonstrate antigenic properties in materials whose protein has been completely split up into amino-acids have usually been unsuccessful. At the stage of proteoses, however, there may be a certain amount of antigenic action; according to Fink (1919) it is the smaller molecules, i.e. those less readily salted out by ammonium sulphate, with which the antigens are associated. Ford (1913) stated that the antigenic constituent of the hæmolysin of the higher fungus *Amanita phalloides* was a glucoside, but this has remained practically an isolated observation. It has been shown repeatedly, however, that an antigen of the usual type may give rise to an antibody which will react *in vitro* with non-protein constituents of the antigen. Thus Forssman's antibody reacts with lipoids extracted from the corresponding antigen (Chapter IX); other examples are considered later (Chapter VII). These non-protein substances

when isolated do not by themselves cause antibody production *in vivo*; therefore Landsteiner has classed them as 'haptens' in contrast to true antigens. It is significant, however, that when mixed with a suitable foreign protein the haptens become reactivated into antigens (Chapter IX), and according to Sachs, Klopstock and Weil (1925) rabbits produce antibody to the lipins of their own kidney tissue when the latter are injected along with pig's serum (see Weil, 1926). According to Meyer (1928), certain lipins—those of tape-worms—act as antigens *in vivo*. Klopstock and Witebsky (1927) made a similar observation with alcoholic extracts of bacteria, but attributed the result to the presence of other substances, since they found such bacterial extracts capable of reactivating haptens into antigens.

Although the first discovered antigens were toxins (Behring and Kitasato, 1890), it was soon shown that the poisonous character was not essential; in fact, the production of severe toxic effects in an animal, by paralysing the reacting mechanism, may obscure the antigenic nature of the substance injected. Accordingly, substances which are practically devoid of toxic properties may act as powerful antigens, e.g. foreign red corpuscles, and the same is true of toxoids, i.e. toxins which have been detoxicated by suitable means (Chapter IV). Again, among substances belonging to the class of antigens some are much more effective as developers of antibodies than others, e.g. the stromata of ox's red corpuscles are a powerful antigen in the rabbit, but haemoglobin or globin is a very weak one (see Browning and Wilson, 1920), and the thermo-labile haemolytic toxin of streptococci appears to be altogether devoid of antigenic properties (McLeod, 1912). The naturally occurring substances used as antigens are probably always mixtures, and apparently in certain cases one antigenic constituent may inhibit the action of another, so that when both are introduced together into an animal antibody is developed only to the former (Chapter IX). Although by no means an invariable occurrence (see Huntoon and Craig, 1921), this 'competition of antigens' complicates further the problem of antigenic action (see Witebsky, 1928). An effective antigen appears to stimulate the tissues in some way and the *ictus immunisatorius*, as Ehrlich and Morgenroth (1900) called it, may be manifested by an obvious histological reaction which favours antibody production in certain cases. This may account for the observation that diphtheria toxin is a much more effective antigen in an animal already partially immunized when it is injected subcutaneously than when given intravenously (Dean, 1908; Madsen, 1923), since in the former case a marked local inflammatory reaction is produced. Of similar significance is Bruck's (1904) observation that of two specimens of tetanus toxoid which both actively fixed antitoxin, the one which was still slightly toxic developed antitoxin on injection, whereas the other, which was quite non-toxic, failed to lead to antitoxin formation. Pfeiffer and Friedberger (1902) also had previously drawn attention to an example of the relationship between virulence and antigenic action. They found that a single small dose of a highly virulent culture of *V. cholerae*, when injected

intravenously into a rabbit caused marked development of bacteriolytic antibody ; with less virulent cultures there was a definite parallelism between virulence and antibody production, avirulent organisms being practically devoid of action.

According to Ehrlich's view, the chemical constitution of the antigen is the factor which determines the specificity of the antibody response and this is confirmed by the observations of Obermayer and Pick and especially of Landsteiner and his co-workers on synthetic antigens (Chapter VII). These develop antibodies whose specificity depends upon the chemical structure of the non-protein moiety of the antigen, and which react with this moiety *in vitro* (see Landsteiner, 1929). But the influence of physico-chemical factors on antigenic action, such as degree of dispersion depending on the electrical charge, may also be important (see Tamari, 1928). The amount of antigen which is required to stimulate antibody formation may be very minute ; as indicated by the delicate anaphylactic test, one twenty-millionth of a gramme of crystallized egg-albumin has been found effective by Wells (1911). This is also shown by experiments in which antibody production has occurred in spite of excision after a very brief interval—minutes or seconds—of the tissue into which the antigen was injected (Oshikawa, 1922 ; Reitler, 1924).

It follows from what has been said above that antigenic action is in general exerted by foreign proteins when brought into direct contact with the tissues—this 'parenteral' introduction being essential, since in the alimentary tract as a rule antigens either are destroyed by the digestive ferment s or fail to be absorbed.

The list of antigens is a very extensive one and comprises substances in the particulate state as well as in solution. The following may be mentioned : bacteria both pathogenic and non-pathogenic, and soluble bacterial derivatives such as toxins and aggressins ; higher plant products including seed-proteins and the phytotoxins, ricin, abrin, crotin and robin ; protozoa and higher animal tissue constituents in the form of cells—red blood corpuscles, leucocytes, blood-platelets, sperm, parenchyma of organs such as liver and kidney, lens—or in solution—serum or isolated serum-protein fractions, milk, the proteins of white of egg, venom of snakes, toad, scorpion, &c. It is doubtful, however, whether ferment s belong to the class of antigens.

#### *Recognition of Antibodies.*

It is convenient to describe as antibody the specific alteration produced in the blood-plasma or serum of an animal which has reacted to an antigen, but it must be clearly understood that the term implies merely the acquisition or enhancement of special properties, and that it does not correspond with a chemically defined substance. Any other changes which have been detected in the constitution of the serum of immunized animals, such as increase in the globulins (see Ledingham, 1907), are probably referable to the general disturbances in metabolism produced by immunization (see Glaessner, 1906).

In considering the nature of antibodies it must be mentioned at the outset that they are often present in the serum of normal animals under conditions which seem to preclude any specific response to an antigen or even the inheritance of such a character, for instance the presence in guinea-pig's serum of a haemolytic immune-body for ox's red corpuscles. These natural antibodies do not differ essentially in their properties from the immune antibodies except that the former tend to be present in small amount and to be relatively thermo-labile (Sachs, 1902), also they may show a comparative lack of specificity (p. 219).

An antibody is recognized by the effects which follow its interaction with the corresponding antigen. Some of these are readily observed *in vitro*, e.g. agglutination; others, such as antitoxic action, are detected by biological tests in the living animal; again, the effect of bacteriotoxins on organisms is not seen until the latter are placed in contact with living leucocytes, when phagocytosis takes place. For the occurrence of bactericidal action, bacteriolysis and haemolysis, the co-operation of the normal blood-constituent, complement, is required in addition to the immune-body; these phenomena can be observed both *in vivo* and *in vitro*. Antibodies are designated according to the nature of the effects which they produce, as already stated. The essential phenomenon, however, is the combination of the antigen with the antibody; the indicators of this combination are often secondary changes which are brought about by non-specific agencies. Thus, Bordet (1899) originally showed that agglutination of bacteria by an antiserum occurred only in the presence of salt, whereas fixation of the agglutinin by the organisms was independent of the electrolyte. Again, cholesterol causes the intensification of certain lipoid-precipitation and complement-fixation reactions, e.g. in the case of heterophile antibody (Chapter IX). Hartley (1925) showed that antitoxic serum extracted with alcohol and ether in the cold no longer flocculated when mixed with toxin in Ramon's test (Chapter IV), although the serum after such treatment had lost none of its capacity to neutralize toxin as tested *in vivo*. On the other hand, similar extraction of an agglutinating antiserum for *B. typhosus* and of haemolytic immune-body did not affect their action. In many instances antibody confers upon the corresponding antigen an affinity for complement or intensifies such an affinity if already present. Several consequences may then ensue and these can be studied *in vitro*. Bacteriolysis may take place, as with *V. cholerae*, which breaks up into granules and finally undergoes solution; similarly, in the case of red corpuscles a poisonous effect is produced which results in haemolysis, the diffusion out of the haemoglobin, the stromata not being destroyed but their physical characters undergoing alteration (Chapter X). Cytolytic antisera cause morphological changes in cells which can be well studied in tissue cultures (Niven, 1929). Some bacteria and cells are killed without undergoing morphological changes. In many cases, however, no obvious effects are produced by the interaction of antigen, immune-body and complement, but the complement becomes 'fixed' or is rendered inactive,

as appears when subsequently some indicator of complement action is introduced into the mixture, e.g. red blood corpuscles sensitized with the corresponding immune-body, which remain intact under these circumstances (Bordet and Gengou, 1901). Many attempts have been made to demonstrate the reactions between antigen and antibody by means of relatively simple indicators. Thus Weichardt (1908, 1910) stated that on placing a dilute solution of acid in contact with dilute alkali, so that a sharp interface resulted, the diffusion of the fluids was accelerated when antigen was present in the acid and the corresponding antibody had been added to the alkaline solution, both being highly diluted. Afterwards the conditions were modified so as to make this 'epiphanin' reaction more obvious. However, the claims as to its delicacy and reliability have not been upheld by later work (see Reich, 1913). Again, Ascoli (1910) recorded that a mixture of antigen (bacterial extract) with the corresponding antiserum, after a period of incubation, showed a reduction of surface tension as compared with that of the mixture when recently prepared or of a corresponding mixture of serum with salt solution instead of the bacterial extract. The drop method with Traube's stalagmometer was used for measuring this 'meiostagmin' reaction. It was found further that heating the serum for half an hour at 56° C. did not affect the reaction and also that similar results were obtained when instead of the watery extract a dilution of an alcoholic extract of the organisms was used. Gouwens (1922) observed that the meiostagmin reaction, as carried out by various methods including that of Du Nouy, failed to show the presence of antibodies in an antiserum of high titre to *B. paratyphosus* B. Instead of the specific antigen artificial mixtures of fatty acids or lipoidal substances have been employed in such experiments and it has been found that serum from a variety of conditions may react positively (see Blumenthal, 1916; Loeb, 1923). Thus, the existence of a direct relationship between this reaction and the presence of antibodies in the serum is doubtful.

#### *Phenomena of Combination of Antigen and Antibody.*

When an antigen and the corresponding antibody are brought into contact actual combination occurs. This originally appeared from the work of Ehrlich (1897<sup>1</sup>) and of Martin and Cherry (1898) in the case of antitoxin. Ehrlich showed that the agglutinating action of ricin on blood corpuscles *in vitro* was abolished by the addition of sufficient antiricin serum and that neutral mixtures as determined by this method were without pathogenic action on mice. Martin and Cherry found that whereas the toxicity of a neutral mixture of thermo-stable snake-venom with antivenin could be restored by heating after a short interval, this was no longer possible when the mixture had stood for some time. Where the antigen is particulate fixation of the antibody can be readily demonstrated, as Ehrlich and Morgenroth (1899) first showed for haemolytic immune-body and Bordet (1899) for bacterial agglutinin and bacteriolytic

immune-body. The cells and antiserum are placed in contact in suitable proportions and after a time are separated by centrifuging, the last traces of serum being removed from the cells by repeated washing with saline. It is then found that the treated serum is deprived of antibody. At the same time the red corpuscles or bacteria are so altered that under suitable conditions they show the changes characteristic of antibody action ; thus, susceptible red corpuscles or bacteria treated with immune-body are sensitized to the action of an appropriate complement, which brings about their lysis at 37° C. Again, bacteria subjected to an agglutinating antiserum clump in the presence of salt ; or organisms treated with bacteriotropins become phagocytosed when brought into contact with leucocytes, &c.

It must be emphasized that antigens and antibodies may also be fixed non-specifically under conditions favourable for adsorption. Thus various finely divided particles fix toxins ; also haemolytic immune-body is removed by contact with heterologous red corpuscles if the latter have been treated previously with osmic acid (Szily, see Kosakai, 1920).

The quantitative relationships exhibited by the combination of antigen and antibody and also the influence of physical and chemical agencies have been extensively investigated. Ehrlich (1897<sup>2</sup>) showed that in certain cases, e.g. the neutralization of diphtheria toxin effected by adding the corresponding antitoxin *in vitro*, the minimum amount of antiserum required to neutralize a given quantity of toxin can be accurately determined by injecting the mixtures into guinea-pigs and that the ' law of multiples ' holds with considerable exactitude. He concluded, therefore, that a molecule of toxin combines firmly with a definite and invariable amount of antibody. The rate of combination is accelerated by increasing the concentration of the reagents or by raising the temperature. When the conditions of experiment are varied, however, differences appear which have been the source of much controversy. Thus, if the amount of antitoxin is determined which is just sufficient to neutralize a given quantity of toxin, say 100 minimum lethal doses, it will be found that much more than one further dose of toxin usually requires to be present before the mixture contains a free lethal dose—the 'Ehrlich phenomenon'. This result has been interpreted by Ehrlich as evidence that a 'toxin' contains molecules possessed of relatively low toxicity and weak combining affinity (epitoxoid or toxone) in addition to those which are highly toxic and combine actively. According to Arrhenius and Madsen (1904<sup>1&2</sup>; see also Arrhenius, 1907), however, antitoxin combines with both toxin and toxoid in conformity with the law of 'mass action' which is exhibited by substances of weak combining affinity. Another striking phenomenon is that a fixed amount of toxin requires less antitoxin to neutralize it when the antitoxin is added in fractions than when it is added all at once ; similarly, a fixed amount of antitoxin neutralizes more toxin when the latter is added all at once than when the toxin is added in fractions—the 'Danysz phenomenon' (1902) (for a chemical analogy, see Arrhenius, 1908). This varying behaviour

has been compared with the adsorption effects exhibited by various substances, for example dyes and filter-paper, where there are no fixed combining proportions (Bordet, 1903; Biltz, Much and Siebert, 1905; Bayliss, 1906). Further, the existence of negative or positive catalysts in the serum has been assumed in order to explain such observations as that a small fraction of a recently prepared neutral mixture of toxin (arachnolysin) and corresponding antiserum became lytic on dilution when the serum used had been recently obtained, but not after it had been kept for some time (Otto and Sachs, 1906). At present it appears to be impossible to analyse the phenomena completely, but the important influence of the colloid medium in which antigen and antibody react must be taken into account. The 'zones' which are met with in agglutination and precipitation, where excess of the homologous antiserum fails to agglutinate organisms or actually dissolves a precipitate, have their counterpart in reactions between colloids (see Neisser and Friedemann, 1904).

Antigen which has united with antibody is in general not destroyed, as has been shown for long-kept neutral mixtures of diphtheria toxin and antitoxin, where the toxicity can be restored, for example by adding hydrochloric acid (Morgenroth and Willanen, 1907). Similarly the combined antibodies may be recovered by causing them to dissociate from particulate antigens; this may be effected in the case of agglutinins and immune-bodies by raising the temperature or altering the medium (Chapter IX).

The assumption should not be made that the reaction between antigen and antibody occurs as if the antiserum contained no component except the latter. There is evidence that the reaction may be influenced by constituents of the serum whose nature is unknown, but whose properties are capable of modification, as in the case of the natural lytic antibody for guinea-pig's red corpuscles contained in ox-serum, the combining affinity of which is temporarily enhanced by passing the serum through a Berkefeld filter (Yoshinare, 1922). Again, it must not be supposed that quantitative relationships established by experiments *in vitro* will necessarily hold *in vivo*. In the living animal the conditions are favourable for prolonged action and hence for dissociation and recombination of antibodies. This is well illustrated by the experiments of Muir and McNee (1912<sup>1&2</sup>) in which the amount of haemolysis resulting from the introduction of an immune-body for the animal's own corpuscles into its circulation was very much greater than could have been expected from estimations in the test-tube.

#### *Conditions affecting the Development of Antibodies.*

Following Ehrlich's (1891) observation that immunity of mice to ricin, produced by feeding, set in suddenly on the sixth day, the antibody response to injections of various types of antigen has been extensively investigated. The general result is that after a single immunizing dose there is a latent period of several days followed by an almost critical

appearance in the blood of antibodies, which continue to increase until they reach their highest concentration at the end of about a week, when they gradually diminish ; this is well shown in the work of Armstrong (1925<sup>1</sup>) on immunization with pneumococci. By means of a series of injections of suitably graded doses of an antigen administered at appropriate intervals the antibody-content of the serum may be raised to a very high level ; advantage is taken of this especially in the preparation of potent antisera for therapeutic purposes. In addition, the antibody response depends greatly on the individuality of the animal.

An effect which may follow shortly after a reinjection of antigen is a sudden temporary decrease of the antibodies in the blood, as was first pointed out by Brieger and Ehrlich (1893) when examining the antitoxin-content of the milk of a goat immunized with tetanus toxin. This ' negative phase ', exhibited by the blood or milk, is of variable magnitude. Salomonsen and Madsen (1897, 1899) found in horses reinfected with diphtheria toxin that the consequent fall in the antitoxin content of the blood was greater than could be accounted for by the amount required to neutralize the injected toxin as estimated *in vitro* ; further, in spite of repeated large doses of toxin administered at short intervals no additional fall occurred, but instead there was a rise in the antitoxin, as Dean (1899) has observed also. Accordingly the negative phase cannot be wholly due to neutralization of antibody by the injected antigen. An explanation of this phenomenon recently given by Ramon and his co-workers (1929) is that the toxin, by causing massive oedema at the site of subcutaneous injection or congestion of organs, especially the liver, after intravenous injection, leads to depletion of the blood fluids and consequent dilution of the plasma by water.

Once an animal has responded to an antigen its tissues have undergone an alteration in reactivity which appears to be of long duration. Accordingly, when the antibody has sunk to a low level or has entirely disappeared from the blood a fresh stimulus rapidly causes its reappearance. Such a stimulus may be supplied by a further injection of the same antigen, as Dean (1899) showed in the case of horses reinfected with diphtheria toxin after a long interval, and as Dungern (1903) found in the case of development of precipitins in response to foreign proteins ; the antibody then appears or begins to increase after a shorter interval than originally. Also the same dose of antigen when repeated in these circumstances causes a greater production of antibody than followed the original dose, or a minute dose may be effective although in a normal animal it would have had no action, as Cole (1904) showed for typhoid agglutinin in rabbits. But non-specific stimuli may also have a similar, though less intense, effect ; thus Dreyer and Walker (1910) pointed out that after the agglutinin-content of the serum developed in a rabbit by an injection of killed *B. coli* had fallen markedly, an injection of a killed culture of other organisms (staphylococci) caused a fresh rise (see also Khanolkar, 1924). Similar results are observed in the human subject, e.g. a paratyphoid

B infection in an individual previously vaccinated with *B. typhosus* causes a reappearance in the blood of specific typhoid agglutinin (Mackie and Wiltshire, 1917). Walbum (1921, 1923) with Mörch (1923) again, has shown that injections of various metallic salts, e.g. manganese chloride, may cause a temporary increase of antibody in the blood of immunized animals, although the inconstancy of this result according to other workers has led to the conclusion that factors still undefined must be concerned (see Mackie, 1925). Therefore, while the antigen supplies the essential or specific stimulus to antibody formation other factors of a non-specific nature may have an important influence on the process.

Antibody production is not inherited from an actively immunized father (Ehrlich, 1892; Ehrlich and Hübener, 1894; Bulloch, 1902). Ehrlich (1892) showed in the case of mice that the young of a non-immune mother when suckled by an actively or passively immunized female rapidly absorbed into their blood the antibodies contained in the milk. In goats and cattle it is the colostrum which is specially effective in transferring the antibodies to the young (Famulener, 1912; Little and Orcutt, 1922) and newborn calves also absorb agglutinins for *Br. abortus* when fed with the serum of immunized cows (Smith and Little, 1923). Mason, Dalling and Gordon (1930) found also in the case of the newborn calf, foal, lamb and pup that after feeding with heterologous serum containing antitoxin absorption of the latter into the blood occurred. On the other hand, antitoxin contained in homologous serum was practically not absorbed when fed to foals 3 and 5 weeks old (Römer, 1909). A transfer of antibodies from the actively or passively immunized mother to the foetus through the placenta has been shown to occur in certain species, e.g. man, guinea-pig and rabbit, but not in others, such as cow and sheep (see Ratner, Jackson and Gruehl, 1927); in the horse a positive result was obtained by Ransom (1900). The fact that the relative proportions of antibody in the serum of the foetus and the actively immunized mother may vary widely suggests that the transfer is not merely passive (Learnmonth, 1924). Newborn animals have little capacity to respond to antigens by the production of antibodies, as appears clearly from Famulener's (1912) experiments in which kids were injected subcutaneously with sheep's red cells.

#### *Nature of Antibodies and Theories regarding their Origin.*

In addition to being present in the plasma and hence in serum and lymph, antibodies appear in other body fluids, but they are abundant only in the milk (Brieger and Ehrlich, 1893; Bulloch, 1902). It is noteworthy that antibodies scarcely appear in the cerebrospinal fluid when the meninges are in a normal state (Taylor, 1917), or even where there is meningitis due to *Meningococcus* (Davis, 1905; see Cruickshank, 1924).

The chemical nature of antibodies is discussed in Chapter VII; it may be stated, however, that nothing definite is known, except that they are associated with one or other of the globulin fractions which may be obtained by salting out the serum. Either antibodies are colloidal in

nature or are bound to colloids, since they do not dialyse or pass through filters which retain colloids (Brodie, 1897). In the case of dissociated *Pneumococcus* antibody, Huntoon, Masucci and Hannum (1921) found that the activity of the solution was not removed by dialysis or digestion with trypsin nor was it extracted by ether, and the solubility did not correspond with that of the globulins, hence they concluded that antibodies are molecules of large size, but are not lipins or any of the ordinary serum-proteins. Also, according to Flaum (1928), rabbits injected intraspinally with red corpuscle stromata or bacteria may develop haemolytic immune-body or agglutinin respectively in their cerebrospinal fluid, although globulins are not demonstrable.

Antibodies are relatively thermo-stable, at least as compared with complement, and withstand heating at 56° C. or even higher temperatures for an hour or longer; according to Klingenstein (1929, 1930), however, certain lipoidophile antibodies are damaged at a lower temperature than those which react with protein antigens. Physical or chemical agencies which denature proteins tend in general to cause deterioration or destruction of antibodies. Also, when fluid serum is kept for a long time at ordinary temperatures the contained antisubstances diminish; in very dilute solutions deterioration may be much more rapid. In the frozen state without the addition of antiseptics, &c., or when dried and kept *in vacuo*, cool and in the dark, serum retains its antibodies unchanged for indefinite periods.

The mode and site of origin of antibodies are obscure and have given rise to speculations many of which are only of historical interest. Of fundamental importance is the fact that antibodies appear as a sort of secretion in the blood-plasma, the quantity developed being often enormously greater than could be fixed by the amount of antigen injected, e.g. in the case of toxin and antitoxin. Evidence for this secretory formation has been obtained by bleeding immunized animals after the injections of antigen have been stopped and when the antibody content of the serum is no longer rising. The content of the regenerated plasma in antibody after extensive bleedings may then reach such a level as is explicable only by its being new formed (Roux and Vaillard, 1893; see also Salomonsen and Madsen, 1898; O'Brien, 1913<sup>1&2</sup>, 1914). It has been concluded, however (Mills, 1925), that in animals recently immunized the antibody which appears in the circulation after bleeding may be due to a redistribution of antibody between the tissue fluids and the blood.

In the early stages of immunization of rabbits with killed cholera bacilli, Pfeiffer and Marx (1898) found bactericidal antibodies more abundant in the spleen, bone-marrow and lymph glands than in the circulating blood; but they failed to recover antisubstances from the leucocytes obtained from the blood or from pleural exudates induced by the injection of aleuronat. Since there was no evidence that in passively immunized animals the above-named tissues accumulated the antibody, it appeared that it must be formed there in active immunization. Van

Emden's (1899) results were confirmatory as regards agglutinin-formation. Dungern (1903) observed the development of precipitin in the aqueous humour of the eye of a rabbit after an injection of foreign protein into the anterior chamber ; precipitin did not appear in the blood till later and it was absent from the aqueous humour of the other eye. The experiments of Oshikawa (1922) in which the transplantation of several small areas of skin from the ear of an actively immunized rabbit into a normal one led to the appearance of antibody in the blood of the latter animal are also highly suggestive of the local production of antibody. In general, however, it has not been possible to extract from any tissue or organ a quantity of antibody so considerable as to prove that one is dealing with a special site of its production (see Rath, 1889 ; Howell, 1928). Recently attempts have been made to identify the reticulo-endothelial system as the site of formation of antibodies, but as yet the evidence for this cannot be regarded as conclusive (compare Bieling, 1923, and Rosenthal, Moses and Petzal, 1924). According to Carrel and Ingebrigsten (1912), haemolytic immune-body for goat's corpuscles is formed in five days in tissue cultures *in vitro* of guinea-pig's bone-marrow, with or without lymph gland, to which goat's red cells have been added. Similarly, Schilf (1926) has recorded the formation of antibody in spleen cultures to which killed El Tor vibrios had been added, the evidence resting upon positive results of Pfeiffer's reaction *in vivo* when the fluid from the cultures was injected along with living vibrios.

Ehrlich (1897<sup>2</sup>, 1900, 1901), in order to account for antibody production, framed a hypothesis known as the receptor or side-chain theory, which affords the only rational explanation advanced so far. The underlying observations are that an antigen such as a toxin when injected into the circulation disappears from the blood and that certain toxins can be shown to combine with tissues *in vitro*, e.g. the fixation of tetanus toxin by the central nervous system (see Noon, 1907). Ehrlich supposed, therefore, that the antigen molecules possess chemical groups, the haptophore groups, whereby they combine with corresponding groups in the protoplasm of the tissue-cells, which resemble the reactive side chains of organic chemical compounds and which he called 'receptors'. This combination is regarded as essential before the poisonous action, considered to be due to a 'toxophore' group of the toxin, can occur. It is assumed that as a result of this combination the cell receptors become saturated and so cease to be available for carrying on the functional requirements of the protoplasm ; at the same time the saturation of the receptors acts as a stimulus to the cells, provided that toxic action has not interfered with their reacting power. Then as a result of the stimulus the cells regenerate receptors equivalent to those which have been saturated ; further, in accordance with the general principles of hypertrophy the new receptors are produced in excess. It is conceived that numbers of these new-formed receptors cease to remain attached to the protoplasm of the cells, i.e. sessile, and become free in the blood-plasma ; it is these free receptors which are believed to constitute antibodies. So long as the crucial test of

recovering antibodies from blood-free tissues is not satisfied the hypothesis lacks proof. At the same time this failure does not necessarily discredit the theory, since chemical methods for recovering the constituents of protoplasm are as yet comparatively undeveloped. Ehrlich believed that the receptor mechanism of the cells was the normal provision for their assimilating foodstuffs ; it is to be noted, however, that in the procedure of immunization foreign proteins come directly into contact with the tissues, and this is a definitely abnormal condition. Therefore acquired antibody formation may be regarded as a biological reaction to the intrusion of substances with foreign specificity.

*The Antigenic Properties of Receptors combined with Antibody.*

It appears, from the foregoing considerations, that the receptor which combines with antibody *in vitro* and the specific component of the antigen which develops antibody *in vivo* are identical. According to this hypothesis one should expect that when the receptors of an antigen are saturated with antibody the treated antigen will be deprived of its capacity to develop antibody. Rehns (1901) found that neutral mixtures of toxin and antitoxin failed to stimulate antitoxin production, but Park (1903-4) obtained formation of antibody under similar conditions, and Babes (1895), who originally investigated the question, had also recorded this result. In Park's experiments the amount of antitoxin formed was only slight, however, when the toxin was injected along with excessive amounts of antitoxin. The test can be readily made where the antigen is particulate, and in accordance with the theory Dungern found that the injection into rabbits of ox's red corpuscles sensitized with immune-body failed to stimulate antibody production. Later workers, however, have obtained variable results (see Dungern, 1903) ; for example, Neisser and Lubowski (1901) found that typhoid bacilli, after treatment with great excess of agglutinating antiserum, were still capable to some extent of developing agglutinins in a proportion of rabbits. Sachs (1901) obtained a similar result as regards production of immune-body on injecting sensitized foreign red cells ; this has been confirmed by Iijima (1925), who has been able by this means to distinguish the heterophile receptors from those which do not correspond with Forssman's antibody. Accordingly, the antigenic power is frequently much diminished but not completely neutralized and this may be attributed either to the fact that it is impossible to saturate the receptors of the antigen completely in the test-tube or to the occurrence of a certain amount of dissociation of antibody from the receptors after the mixture has been injected. Of course, where the receptors are only partially saturated one would expect a more marked production of antibodies than where they are fully saturated. Besredka (1910) applied this principle in the preparation of 'sensitized vaccines'—the organisms are treated with homologous antiserum, then washed and used for injection. It is claimed that by such a procedure toxic effects are diminished while a satisfactory immunity response occurs. Armstrong (1925<sup>2</sup>) has investigated the question

in the case of sensitized pneumococci ; he concluded that *in vivo* the antibodies were rapidly dissociated, and that active immunity was developed, but antibody production was less in degree and occurred more slowly than was the case when untreated organisms were injected. With toxins it has also been found advantageous in certain circumstances to mitigate the toxic effects by the addition of antitoxin. Thus, partially neutralized mixtures have been used for the purposes of producing active immunity against diphtheria. Again, for the protection of animals the addition of antiserum to living viruses has had the effect of controlling the pathogenic action of the latter, while permitting active immunity to develop.

#### *Specificity.*

Ehrlich's hypothesis aims at explaining immunological specificity. The significance of specificity in this sense appears from the following examples. An antiserum developed by injections of diphtheria toxin neutralizes the toxin of every culture of diphtheria bacilli, but towards the toxins of other organisms, e.g. *B. tetani* or *B. botulinus*, it behaves like normal serum. Again, an antiserum versus *V. cholerae* leads to bacteriolysis of the homologous culture and also of different strains of this organism, but towards other vibrios it behaves in this respect like normal serum. In the instances just mentioned the specificity is practically absolute. On the other hand, a precipitin for human serum has some precipitating action on monkey's serum, but towards serum of other species its action scarcely differs from that of a normal serum. Here there is a 'group reaction', since the antihuman serum reacts with the serum of a zoologically related species. Such group reactions are frequently met with ; for instance, the lytic immune-body produced by injecting ox's red corpuscles into a rabbit leads also to lysis of sheep's red corpuscles, but its action on the latter is much weaker than on the former. In the case of certain bacteria, e.g. the *Salmonella* group, it is common to find that an agglutinating serum acts powerfully on organisms other than the homologous one ; and complicated relationships between different members of the group have been made out by studying the combining properties of antisera for different strains when tested with various cultures. In contrast to the above observations, examples are also met with of strains of organisms which as judged by their general characters are all of one kind and yet possess different antigens. Thus *B. botulinus* falls into two distinct types, which have been named A and B, as regards the antigenic properties of its toxin. Although the toxins of both types have the same pathogenic action, that of type A is neutralized only by the corresponding antitoxin and not by an antiserum to type B toxin, and vice versa. Again, on the basis of agglutination with antisera derived from rabbits meningococci can be separated into four types (Gordon, 1917). On the other hand, as in the case of the Forssman antigen, the same receptors may occur in the tissues of a wide variety of animals, their distribution having no correspondence with zoological relationships (Chapter IX).

Since organisms and their products, tissue-cells, natural protein solutions like serum, &c., are of extremely complex composition, it might be expected that they would contain a mixture of antigens, as Ehrlich (1898, 1901) originally postulated. This has been demonstrated in the morphological sense in the case of the agglutinogens of certain motile bacilli, such as *B. typhosus*, in which a thermo-labile flagellar antigen and a thermo-stable somatic antigen have been distinguished. Also, the non-motile bacilli of the Flexner-Y group contain varying proportions of at least four different agglutinogens. The red corpuscles of the sheep contain two types of antigen, one of which in rabbits develops an ordinary immune-body, which has, however, an affinity also for ox's corpuscles. The other is the Forssman lipoidal antigen, the corresponding antibody to which does not combine with ox's corpuscles although it reacts with tissue constituents belonging to a number of animals. According to the important observations of Dakin and Dale (1919) the egg-albumins of different species, which can be differentiated by anaphylactic tests, are also distinguishable by differences in the arrangement of the constituent amino-acids in the protein molecules. Therefore the results of a large series of observations are in agreement with Ehrlich's chemical conception of specificity, according to which an antibody represents the response to an antigen of particular chemical structure. According to Locke, Main and Hirsch (1926) the specificity of the antibody is due to its actually containing a 'binding substance' derived from the injected antigen; but the evidence in support of this view is not convincing.

Sometimes an antigen is practically restricted to a given species of animal or bacteria and then great value attaches to its reaction with the corresponding antibody as a means of identification, e.g. the flagellar agglutination of *B. typhosus* for identifying that organism, and the precipitation and complement-fixing reactions for determining the species of blood-serum. The delicacy of such reactions may be very great; thus, by the use of a specific antiserum from the rabbit it is possible to obtain fixation of complement with 0.00001 c.cm. or less of human serum.

Another practical application of the specificity of the interaction between antigen and antibody is known as Castellani's (1902) reaction. This depends upon the fact that when an antiserum to a particular organism agglutinates several other organisms treatment with the homologous bacteria removes all the agglutinins, whereas treatment with the heterologous organisms removes only the group-agglutinins and the treated serum still agglutinates the homologous bacteria.

It has already been noted that antibodies may occur normally without the stimulus of a specific antigen. Certain conditions of disease appear to excite the development of such natural antibodies and their appearance in the blood may possess great importance in diagnosis. This is the case with the agglutinins for *B. proteus* (strain X-19, &c.), which develop so constantly in the blood in typhus fever as to constitute a valuable

means of recognizing this disease (Weil-Felix reaction), although the ætiological relation of this organism to the disease is most doubtful.

Since the first stage in the process of antibody formation is supposed to consist in the combination of the haptophore group of the antigen with preformed receptors of the protoplasm, the question arises as to whether distinct receptors must exist in an animal's tissues for every antigen to which it can develop antibodies. Investigations on natural antibodies, such as those of Landsteiner (1909) with Reich (1907) and others on haemagglutinins, and of Dunlop (1928) on bacterial complement-fixing antibodies, indicate that high specificity is frequently lacking in natural antibodies and suggest that there is a more or less unspecialized prototype from which the highly specific antibodies evolve in the course of immunization (see also Mackie and Finkelstein, 1930). Thiele and Embleton's (1914) observations on the development of haemolytic immune-bodies (Chapter IX) also point in the same direction. This conception obviates the need for supposing a specific pre-existing receptor to suit every antigen. In conclusion it may be said that Ehrlich's theory in its general sense, with minor modifications, has been widely accepted as a working hypothesis. Of experimental evidence adduced against it that of Manwaring (1928) rests on anaphylactic phenomena, the interpretation of which appears to be in need of further elucidation.

#### *Characters of Antibodies depending on their Source.*

Antibodies possess also specific characters which depend upon the species of animal which has developed them. This appears clearly from observations on the properties of haemolytic immune-bodies obtained by injecting the same species of red corpuscles into different animals (Muir, 1912). Although the resulting antibodies may appear to combine with the same receptors of the corpuscles, the effects may vary widely, as indicated by the susceptibility of the sensitized red cells to different complements (Chapter IX). One of the most striking proofs of the influence of the species of origin of antibodies in determining their characters is the fact first noted by Bordet (1904) that on injecting normal rabbit's serum into another animal, e.g. the guinea-pig, an anti-immune-body develops which has the property of hindering the fixation of complement by red cells sensitized with an immune-body from the rabbit. Thus the anti-immune-body is specific for immune-bodies derived from the rabbit and it acts on these irrespective of the species of red corpuscles used to develop them. Whether in virtue of the development of anti-antibodies of this nature or simply because of association of antisubstances with the proteins (see Pfeiffer and Friedberger, 1904), it is found that passive immunization of an animal with antiserum derived from another species lasts a relatively short time. The antibodies disappear from the blood within a week (Levin, 1909); after repeated injections the rate of disappearance of antibodies is greatly accelerated, as Glenny and Hopkins (1924) showed in the case of a rabbit injected intravenously at weekly

intervals with diphtheria antitoxin from the horse. Another character of antisera which depends upon the species yielding them is illustrated by agglutinins for meningococci (Fildes, 1920). As is well known, these organisms can be separated into types by the use of agglutinating antisera from rabbits, especially when young animals are injected and the sera are obtained at an early stage of immunization. However, the antiserum derived from a horse which had been repeatedly inoculated with a single strain agglutinated all types of meningococci practically to an equal degree. It is difficult to decide in such cases whether the differences in the antisera represent a varied response on the part of the animals to the same antigen, or whether there are multiple antigens, some of which exert a stronger stimulus in one species than in another. The part which may be played by the competition of antigens has already been referred to.

*Multiplicity of Antibodies : Avidity.*

The question arises whether the different manifestations of antibody action are due to different antibodies, i.e. whether agglutinins, cytotoxins, immune-bodies, &c., have a separate existence. It is, of course, general experience that a given antiserum may lead to one of these effects in marked degree and fail to produce others, but so long as antibodies cannot be isolated in the pure state a particular result may mean that the conditions are specially favourable for its occurrence; and there is no clear evidence that a multiplicity of antibodies is developed by an animal in response to a single antigen. It was long held that neutralization of toxin by antitoxin was unaccompanied by any other immunity reaction, but Ramon (1923) showed that when the reagents were brought together in suitable proportions at high concentration, precipitation occurred and Dean (1927) has demonstrated also complement fixation. Again, the action of complement along with immune-body is usually a lytic one, as is strikingly shown by its dissolving the precipitate obtained when Forssman's antibody acts on the corresponding lipoidal extracts (Taniguchi, 1921). But in special circumstances complement may bring about agglutination of sensitized corpuscles (Chapter X). A separate identity of certain of the antibodies in an antiserum is, however, suggested by the work of Ruppel, Ornstein, Carl and Lasch (1923), because, on fractioning a cholera antiserum by the method of electro-osmosis, the separated globulin components were found to have different antibody properties attached to them.

There is definite evidence that the antibody molecules in an antiserum are not all uniform in character. Müller (1911) stated that the agglutinin of specific antisera was not a homogeneous property (see also Barikin and Friese, 1926). Thus, after absorbing with a fixed quantity of homologous organisms similar amounts of different specimens of specific antiserum, as measured by the agglutinating titre of the dilutions used, the agglutinating power of the treated sera was found to vary (Busson, Müller and Rintelen, 1909). Similarly, by repeated treatment with fresh doses of the

homologous organisms it was shown that the agglutinin of a given anti-serum consisted of molecules of varying avidity. Differences in 'avidity' of antibodies, leading in the case of antitoxins to differences in the rate at which they neutralize the same toxin, have been indicated also by the investigations of Kraus (1924) and his co-workers (see Barikine, 1912). Further, the immune-body present in a haemolytic antiserum at different stages of immunization has been found to vary in its capacity for leading to fixation of complement when the red cells are sensitized with multiple doses (Chapter X).

The avidity of antibody, which may be of great importance therapeutically, was clearly shown by Madsen (1899) *in vitro* in the case of tetanolysin. A lytic dose of this toxin is rapidly fixed by the red cells, but some time elapses before lysis begins. If during this latent interval the corpuscles are treated with antiserum lysis can be prevented, because the affinity or avidity of the antitoxin is apparently greater than that of the cells for tetanolysin, and so the combination of the latter becomes dissociated.

## REFERENCES.

ARMSTRONG, R. R., 1925<sup>1</sup>, *Proc. Roy. Soc., B*, **98**, 525; 1925<sup>2</sup>, *ibid.*, **98**, 545.  
 ARRHENIUS, S., 1907, *Immunochemistry*, New York; 1908, *J. Hyg., Camb.*, **8**, 1.  
 ARRHENIUS, S. & MADSEN, T., 1904<sup>1</sup>, *Zbl. Bakter., Abt. I, Orig.*, **36**, 612; 1904<sup>2</sup>,  
*ibid.*, **37**, 1.  
 ASCOLI, M., 1910, *Münch. med. Wschr.*, **57**, 62.  
 BABES, M. V., 1895, *Bull. Acad. Méd.*, Paris, **34**, 206.  
 BARIKINE, W., 1912, *Z. ImmunForsch.*, Tl. I, *Orig.*, **15**, 329.  
 BARIKIN, W. & FRIESE, W., 1926, *Z. ImmunForsch.*, **45**, 191.  
 BAYLISS, W. M., 1906, *Bio-Chem. J.*, **1**, 175.  
 BEHRING, 1890, *Deuts. med. Wschr.*, **16**, 1145.  
 BEHRING & KITASATO, 1890, *Deuts. med. Wschr.*, **16**, 1113.  
 BESREDKA, A., 1910, *Bull. Inst. Pasteur*, **8**, 241.  
 BIELING, R., 1923, *Z. ImmunForsch.*, Tl. I, *Orig.*, **38**, 193.  
 BILTZ, W., MUCH, H. & SIEBERT, C., 1905, *Beitr. exp. Ther.*, Hft. **10**, 30.  
 BLUMENTHAL, N., 1916, *Z. ImmunForsch.*, Tl. I, *Orig.*, **24**, 42.  
 BORDET, J., 1899, *Ann. Inst. Pasteur*, **13**, 225; 1903, *ibid.*, **17**, 161; 1904, *ibid.*,  
**18**, 593.  
 BORDET, J. & GENGOU, O., 1901, *Ann. Inst. Pasteur*, **15**, 289.  
 BRIEGER & EHRLICH, P., 1893, *Z. Hyg. InfektKr.*, **13**, 336.  
 BRODIE, T. G., 1897, *J. Path. Bact.*, **4**, 460.  
 BROWNING, C. H. & WATSON, H. F., 1913, *Int. Clin.*, 23rd ser., **2**, III.  
 BROWNING, C. H. & WILSON, G. H., 1920, *J. Immunol.*, **5**, 417.  
 BRUCK, C., 1904, *Z. Hyg. InfektKr.*, **46**, 176.  
 BULLOCH, W., 1902, *Trans. Path. Soc.*, Lond., **53**, 189.  
 BUSSON, B., MÜLLER, P. TH. & RINTELEN, A., 1909, *Z. ImmunForsch.*, Tl. I, *Orig.*,  
**3**, 217.  
 CALMETTE, A., 1894, *Ann. Inst. Pasteur*, **8**, 275.  
 CARREL, A. & INGEBRIGSTEN, R., 1912, *J. Exp. Med.*, **15**, 287.  
 CASTELLANI, A., 1902, *Z. Hyg. InfektKr.*, **40**, 1.  
 CHARRIN & ROGER, 1889, *C.R. Soc. Biol.*, Paris, 9 s., i, 667.  
 COLE, R. I., 1904, *Z. Hyg. InfektKr.*, **46**, 371.  
 CRUICKSHANK, J., 1924, in *Recent Advances in the Diagnosis and Treatment of Syphilis*, by C. H. Browning and I. Mackenzie, 2 ed., London.  
 DAKIN, H. D. & DALE, H. H., 1919, *Biochem. J.*, **13**, 248.  
 DANYSZ, J., 1902, *Ann. Inst. Pasteur*, **16**, 331.  
 DAVIS, D. J., 1905, *J. Infect. Dis.*, **2**, 602.

DEAN, G., 1899, *Trans. Path. Soc., Lond.*, **51**, 15; 1908, in *The Bacteriology of Diphtheria*, edited by G. H. F. Nuttall and G. S. Graham-Smith, Camb. Univ. Press.

DEAN, H. R., 1927, *J. Path. Bact.*, **30**, 675.

DENYS, J. & LECLEF, J., 1895, *Cellule*, **11**, 177.

DONATH, J. & LANDSTEINER, K., 1904, *Münch. med. Wschr.*, **51**, 1590; 1925, *Ergebn. Hyg. Bakt.*, **7**, 184.

DREYER, G. & WALKER, E. W. A., 1910, *J. Path. Bact.*, **14**, 28.

DUNGERN, E. v., 1903, *Die Antikörper*, Jena.

DUNLOP, E. M., 1928, *J. Path. Bact.*, **31**, 769.

DURHAM, H. E., 1896, *Proc. Roy. Soc.*, **59**, 224.

EBERT, M. K., 1929, *Z. ImmunForsch.*, **63**, 455.

EHRLICH, P., 1891, *Deuts. med. Wschr.*, **17**, 976, 1218; 1892, *Z. Hyg. InfektKr.*, **12**, 183; 1897<sup>1</sup>, *Fortschr. Med.*, **15**, 41; 1897<sup>2</sup>, *Klin. Jahrb.*, **6**, 299; 1898, *Deuts. med. Wschr.*, **24**, 597; 1900, *Proc. Roy. Soc.*, **66**, 424; 1901, *Schlussbetrachtungen in Erkrankungen des Blutes*, by Nothnagel, *Specielle Pathologie u. Therapie*, **8**, Wien.

EHRLICH, P. & HÜBENER, W., 1894, *Z. Hyg. InfektKr.*, **18**, 51.

EHRLICH, P. & MORGENROTH, J., 1899, *Berl. klin. Wschr.*, **36**, 6, 481; 1900 *ibid.*, **37**, 681.

VAN EMDEN, J. E. G., 1899, *Z. Hyg. InfektKr.*, **30**, 19.

FAMULENER, L. W., 1912, *J. Infect. Dis.*, **10**, 332.

FILDES, P., 1920, *Brit. J. Exp. Path.*, **1**, 44.

FINK, E. B., 1919, *J. Infect. Dis.*, **25**, 97.

FLAUM, A., 1928, *Acta Path. Microbiol. Scand.*, **5**, 16.

FORD, W. W., 1913, *Zbl. Bakt., Abt. I, Ref.*, **58**, 193.

GENGOU, O., 1902, *Ann. Inst. Pasteur*, **18**, 734.

GLAESSNER, K., 1906, *Z. exp. Path. Ther.*, **2**, 154.

GLENNY, A. T. & HOPKINS, B. E., 1924, *J. Hyg., Camb.*, **22**, 208.

GORDON, M. H., 1917, *Sp. Rep. Ser. Med. Res. Comm.*, No. 3.

GOUWENS, W. E., 1922, *J. Infect. Dis.*, **31**, 237.

HARTLEY, P., 1925, *Brit. J. Exp. Path.*, **6**, 180.

HOWELL, K. M., 1928, *The newer knowledge of bacteriology and immunology*, ed. E. O. Jordan and I. S. Falk, Chicago.

HUNTOON, F. M. & CRAIG, S. H., 1921, *J. Immunol.*, **6**, 235.

HUNTOON, F. M., MASUCCI, P. & HANNUM, E., 1921, *J. Immunol.*, **6**, 185.

IIJIMA, T., 1925, *J. Path. Bact.*, **28**, 397.

KHANOLKAR, V. R., 1924, *J. Path. Bact.*, **27**, 181.

KLINGENSTEIN, R., 1929, *Klin. Wschr.*, **8**, 1860; 1930, *Z. ImmunForsch.*, **66**, 99.

KLOPSTOCK, A. & WITEBSKY, E., 1927, *Klin. Wschr.*, **6**, 119.

KOSAKAI, M., 1920, *J. Path. Bact.*, **23**, 425.

KRAUS, R., 1897, *Wien. klin. Wschr.*, **10**, 736; 1924, *Z. ImmunForsch.*, **41**, 170.

LANDSTEINER, K., 1909, *Wien. klin. Wschr.*, **22**, 1623; 1929, *Z. ImmunForsch.*, **62**, 178.

LANDSTEINER, K. & REICH, M., 1907, *Z. Hyg. InfektKr.*, **58**, 213.

LEARMONTH, J. R., 1924, *J. Hyg., Camb.*, **22**, 100.

LEDINGHAM, J. C. G., 1907, *J. Hyg., Camb.*, **7**, 65.

LEVIN, E. I., 1909, *Z. ImmunForsch.*, Tl. I, Orig., **1**, 1.

LITTLE, R. B. & ORCUTT, M. L., 1922, *J. Exp. Med.*, **35**, 161.

LOCKE, A., MAIN, E. R. & HIRSCH, E. F., 1926, *J. Infect. Dis.*, **39**, 126.

LOEB, L. F., 1923, *Biochem. Z.*, **136**, 190.

MACKIE, T. J., 1925, *J. Hyg., Camb.*, **24**, 176.

MACKIE, T. J. & FINKELSTEIN, M. H., 1930, *J. Hyg., Camb.*, **30**, 1.

MACKIE, T. J. & WILTSHIRE, H. G., 1917, *R.A.M.C. Jl.*, **29**, 276.

MADSEN, T., 1899, *Z. Hyg. InfektKr.*, **32**, 239; 1923, *J. State Med.*, **31**, 51, 101.

MCLEOD, J. W., 1912, *J. Path. Bact.*, **16**, 321.

MANWARING, W. H., 1928, *The newer knowledge of bacteriology and immunology*, ed. E. O. Jordan and I. S. Falk, Chicago.

MARTIN, C. J. & CHERRY, T., 1898, *Proc. Roy. Soc.*, **63**, 420.

MASON, J. H., DALLING, T. & GORDON, W. S., 1930, *J. Path. Bact.*, **33**, 783.

MEYER, K., 1928, *Z. ImmunForsch.*, **57**, 42.

MILLS, J., 1925, *J. Path. Bact.*, **28**, 579.  
 MORGENTHOTH, J. & WILLANEN, K., 1907, *Virchows Arch.*, **190**, 371.  
 MUIR, R., 1912, *J. Path. Bact.*, **16**, 143, 523.  
 MUIR, R. & MCNEE, J. W., 1912<sup>1</sup>, *J. Path. Bact.*, **16**, 410; 1912<sup>2</sup>, *ibid.*, **17**, 92.  
 MÜLLER, P. T., 1911, *Handb. d. Technik. u. Methodik d. ImmunForsch.*, hrsg. Kraus und Levaditi, I. Ergänzgsbd., Jena.  
 NEISSE, M. & FRIEDEMANN, U., 1904, *Münch. med. Wschr.*, **51**, 465, 827.  
 NEISSE, M. & LUBOWSKI, R., 1901, *Zbl. Bakt.*, Abt. I, Orig., **30**, 483.  
 NIVEN, J. S. F., 1929, *J. Path. Bact.*, **32**, 527.  
 NOON, L., 1907, *J. Hyg., Camb.*, **7**, 101.  
 O'BRIEN, R. A., 1913<sup>1</sup>, *J. Hyg., Camb.*, **13**, 353; 1913<sup>2</sup>, *J. Path. Bact.*, **17**, 425; 1914, *ibid.*, **18**, 89, 423.  
 OSHIKAWA, K., 1922, *Z. ImmunForsch.*, **33**, 297, 306.  
 OTTO, R. & SACHS, H., 1906, *Z. exp. Path. Ther.*, **3**, 19.  
 PARK, W. H., 1903-4, *Proc. New York Path. Soc.*, New Ser., **3**, 139.  
 PFEIFFER, R., 1894, *Deuts. med. Wschr.*, **20**, 898.  
 PFEIFFER, R. & FRIEDBERGER, E., 1902, *Berl. klin. Wschr.*, **39**, 581; 1904, *Zbl. Bakt.*, Abt. I, Orig., **37**, 138.  
 PFEIFFER, R. & MARX, 1898, *Z. Hyg. InfektKr.*, **27**, 272.  
 PHYSALIX, C. & BERTRAND, G., 1894, *C.R. Acad. Sci., Paris*, **118**, 356.  
 RAMON, G., 1923, *Ann. Inst. Pasteur*, **37**, 1001; 1929, *C.R. Soc. Biol., Paris*, **100**, 783.  
 RAMON, G., DESCOMBEY, P. & VALOT, P., 1929, *C.R. Soc. Biol., Paris*, **100**, 786.  
 RANSOM, F., 1900, *J. Path. Bact.*, **6**, 180.  
 RATH, D., 1899, *Zbl. Bakt.*, Abt. I, **25**, 549.  
 RATNER, B., JACKSON, H. C. & GRUEHL, H. L., 1927, *J. Immunol.*, **14**, 249.  
 REHNS, J., 1901, *C.R. Soc. Biol., Paris*, 11 s., **3**, 141.  
 REICH, E., 1913, *Z. ImmunForsch.*, Tl. I, Orig., **18**, 480.  
 REITLER, R., 1924, *Z. ImmunForsch.*, **40**, 453.  
 RÖMER, P. H., 1909, *Z. ImmunForsch.*, Tl. I, Orig., **1**, 171.  
 ROSENTHAL, F., MOSES, A. & PETZAL, E., 1924, *Z. ges. exp. Med.*, **41**, 405.  
 ROUX, E. & VAILLARD, L., 1893, *Ann. Inst. Pasteur*, **7**, 65.  
 ROUX, E. & YERSIN, 1888, *Ann. Inst. Pasteur*, **2**, 629.  
 RUPPEL, W. G., ORNSTEIN, O., CARL, J. & LASCH, G., 1923, *Z. Hyg. InfektKr.*, **97**, 188.  
 SACHS, H., 1901, *Zbl. Bakt.*, Abt. I, **30**, 491; 1902, *Berl. klin. Wschr.*, **39**, 181, 216.  
 SACHS, H., KLOPSTOCK, A. & WEIL, A. J., 1925, *Deuts. med. Wschr.*, **51**, 589, 1017.  
 SALMON, D. E. & SMITH, TH., 1884-6, *Proc. Biol. Soc., Wash.*, **3**, 29.  
 SALOMONSEN, C. J. & MADSEN, T., 1897, *Ann. Inst. Pasteur*, **11**, 315; 1898, *ibid.*, **12**, 763; 1899, *ibid.*, **13**, 262.  
 SCHILF, F., 1926, *Zbl. Bakt.*, Abt. I, Orig., **97**, 219.  
 SCHWARZMANN, L., 1927, *Z. ImmunForsch.*, **51**, 139.  
 SMITH, T. & LITTLE, R. B., 1923, *J. Exp. Med.*, **37**, 671.  
 TAMARI, L., 1928, *Z. ImmunForsch.*, **57**, 85.  
 TANIGUCHI, T., 1921, *J. Path. Bact.*, **24**, 456.  
 TAYLOR, F. E., 1917, *Lancet, Lond.*, i, 418.  
 THIELE, F. H. & EMBLETON, D., 1914, *Z. ImmunForsch.*, Tl. I, Orig., **20**, 1.  
 WALBUM, L. E., 1921, *C.R. Soc. Biol., Paris*, **85**, 761; 1923, *ibid.*, **89**, 1007.  
 WALBUM, L. E. & MÖRCH, J. R., 1923, *Ann. Inst. Pasteur*, **37**, 396.  
 WEICHARDT, W., 1908, *Zbl. Bakt.*, Abt. I, Ref., **47**, 143\*; 1910, *Z. ImmunForsch.*, Tl. I, Orig., **6**, 644.  
 WEIL, A. J., 1926, *Z. ImmunForsch.*, **48**, 81.  
 WELLS, H. G., 1911, *J. Infect. Dis.*, **9**, 147.  
 WITEBSKY, E., 1928, *Z. ImmunForsch.*, **58**, 297.  
 WRIGHT, A. E. & DOUGLAS, S. R., 1903, *Proc. Roy. Soc.*, **72**, 357.  
 YOSHINARE, N., 1922, *J. Path. Bact.*, **25**, 153.

## CHAPTER VII. THE EFFECT OF PHYSICAL AND CHEMICAL AGENCIES ON THE PROPERTIES OF ANTIGENS AND ANTIBODIES.

By P. HARTLEY

(NATIONAL INSTITUTE FOR MEDICAL RESEARCH, HAMPSTEAD, LONDON).

### **Antigens.**

THE study of the effect of physical and chemical agencies on the antigenic properties of living micro-organisms—a problem of practical importance in vaccine and serum therapy—presents peculiar difficulties arising partly from the fact that the true antigen occurs as a relatively small part of a very heterogeneous mixture, and partly because the antigenic structure of the living cell is so very complex.

The discovery that all complete, naturally occurring soluble proteins, whether of animal or vegetable origin, possess antigenic function gave a new direction to the study of this and related problems in immunology. The naturally occurring proteins are, in the main, non-toxic substances, and although they give rise to antibodies with properties different from those, for example, of antitoxins, their fundamental properties as antigens are not essentially different from those of virulent micro-organisms or their toxic products. The physical and chemical changes produced in protein solutions under certain definite and reproducible conditions can be followed, and immunological investigations carried out with natural proteins, and particularly with single proteins purified to the highest degree possible, have added something to our knowledge of the nature and properties of antigens in general.

### **THE EFFECT OF DENATURATION AND COAGULATION ON ANTIGENIC FUNCTION.**

The antigenic properties of many proteins are modified, reduced or altogether lost when subjected to influences which lead to denaturation or coagulation. It is not known whether the different kinds of denatured protein obtained by subjecting solutions of the same protein to different denaturing agencies—heat, light, mechanical shaking, acids and alkalis, alcohol and acetone, alkaloidal reagents, heavy metal salts, &c.—are identical in character, although recent investigations suggest that some of these forms are closely related immunologically; neither is it known whether the reactions involved in the different types of denaturation are the same, or whether in all cases denaturation and coagulation are similarly affected by temperature, reaction and electrolytes.

## EFFECT OF PHYSICAL AND CHEMICAL AGENCIES ON 225 THE PROPERTIES OF ANTIGENS AND ANTIBODIES

Chick and Martin (1910, 1911, 1912) showed that the familiar phenomenon of coagulation by heat is fundamentally a reaction between protein and water and consists of two distinct processes : (i) denaturation of the protein due to the action of hot water, and (ii) the separation of the denatured protein in particulate form—agglutination or coagulation. The latter stage of the process has been studied in greater detail than the former ; yet it is during, or as a result of, denaturation that changes occur in the protein molecule as a result of which its properties are markedly altered. The protein is changed from an emulsoid or hydrophile type of colloid to one having the properties of a suspensoid or hydrophobe colloid. Many observations have been made which show that some change occurs in the actual chemical structure of the protein molecule during denaturation, although no explanation of these changes has yet been advanced which has met with general acceptance. The combining capacity for acids and bases is increased (Chick and Martin), and the isoelectric point is shifted slightly to the alkaline side (Michaelis and Davidsohn, 1911). Denatured and coagulated proteins give an intense sulphydryl reaction (Harris, 1923) and disulphide reaction (Walker, 1925) and undergo oxidation and reduction with glutathione (Hopkins, 1925). Sörensen (1925) was unable to demonstrate the liberation of nitrogen compounds during denaturation, but found an increase in non-protein nitrogen in solution after coagulation. Wu and Wu (1925) observed slight chemical changes during denaturation and advance the view that the same changes occur in heat denaturation as in denaturation by acids and alkalis. Young (1922) found that changes in viscosity, optical rotation, and surface tension occur as a result of denaturation by light and considers that light denaturation is very similar to, if not identical with, heat denaturation. Denatured proteins are more easily digested by certain enzymes than the same proteins in the native state.

It is believed that the chemical changes which occur during denaturation are irreversible, though Anson and Mirsky (1925) have recently suggested that the change is reversible, the reversibility being usually obscured by flocculation of the denatured protein. That some chemical change occurs during denaturation is admitted ; whether the change is in the nature of a dehydration, or a feeble or incipient hydrolysis or a depolymerization is undecided.

The changes which occur on denaturation have an important application in immunology, since the agencies which effect denaturation are also those which influence antigenic function and immunological specificity. The effect of heat on the antigenic activity of proteins is very diverse and varies with particular proteins and the conditions under which they are heated. Solutions of non-coagulable proteins, as was shown by Wells for caseinogen (1908) and  $\beta$ -nucleoprotein (1916), for certain seed proteins (Wells and Osborne, 1915) and for ovomucoid and seromucoid (Lewis and Wells, 1927), may be heated to boiling without coagulation taking

place and still retain their antigenic properties : and solutions of coagulable protein, if heated under conditions which, though causing denaturation, leave the denatured protein in solution, retain antigenic properties, although their activity is usually greatly reduced and specificity may be changed.

The question as to whether proteins which in the native state are active antigens retain any antigenic activity after they have been coagulated and rendered insoluble in water is of interest from the theoretical and practical points of view. There is some evidence that the different manifestations of antigenic activity which a protein normally exhibits are affected in different degrees by heat. The property of sensitizing anaphylactically and of producing anaphylaxis in sensitized animals is markedly affected by heat ; it almost disappears by prolonged heating to 100° C., and if the protein is completely coagulated the property is lost altogether (Wells, 1908). It has been shown, however, that complement-fixing antibodies (Schmidt, 1921) and precipitins (Fujiwara, 1922) appear in the serum of animals after injection of heat-coagulated proteins. Although the claims made by the latter in regard to the rigid specificity of such sera have not been universally admitted, the fact that precipitins are produced to heat-coagulated proteins has been confirmed by Beger (1924), Rosenberg (1926), Nicolas and Katrandjieff (1928), and others. It is generally assumed that an antigen must be soluble in the tissues of the injected animal in order to exert its antigenic function, and it would appear reasonable to assume that the body possesses some mechanism of bringing into solution protein which has been rendered water-insoluble. Wells and Lewis (1924) have recorded that the supernatant fluid from a washed suspension of heat-coagulated, water-insoluble egg-albumin, after long standing in chloroform water, occasionally sensitized guinea-pigs ; and that intraperitoneal injections of suspensions of coagulated proteins sensitize animals to the same uncoagulated protein. Confirmatory results were obtained with complement-fixation tests. They suggest that coagulation by heat is not an absolutely irreversible reaction and that *in vivo* the reaction may proceed more readily. Lewis and Wells (1927) have pointed out that egg and serum mucoids which do not coagulate on boiling and retain antigenic function even when their solutions are boiled, occur in amounts sufficiently large to make them of significance in immunological studies with heated proteins, and it is possible that antibody production may have been due to uncoagulated protein either occluded within the particles of coagulated protein or adherent to them.

It has frequently been shown that heating produces some change in the antigenic structure of proteins. Obermayer and Pick (1906) found that the precipitin for native ox-serum did not react with heated ox-serum, while the precipitin for heated ox-serum reacted with native and heated ox-serum. The 'cocto precipitin', though exhibiting a wider range of reaction was, nevertheless, species-specific. Schmidt (1909, 1912).

in a detailed study of the action of heat and alkali, separately and combined, showed that serum-proteins retain antigenic function after heating under various conditions, but the range of the reaction of the precipitins they engendered was modified as a result of the heating, or the alkali, or both. Alkalized serum, when incubated at 25 to 30° C., gradually lost the property of reacting with a precipitin for native serum, and the rate of inactivation was dependent on the concentration of the alkali and the time of incubation. He also showed that after a certain time of incubation in alkaline solution serum lost its true antigenic function altogether, i.e. no antibodies were produced when such alkali-treated sera were injected into animals. Schmidt, in agreement with Obermayer and Pick, found that although changes in antigenic structure occur when serum is heated, species-specificity is retained. Zinsser and Ostenberg (1914) and Furth (1925, 1926), on the other hand, found that the precipitins engendered by heated proteins are not species-specific. Furth considers the failure to observe this change in species-specificity by Obermayer and Pick and Schmidt to have been due to testing the antisera over too narrow a range of antigen dilutions. He found that crystalline egg-albumin, when heated to 100° C. for 5 minutes, behaved serologically like a new protein. Furth also showed that rabbits did not produce antibodies to heated rabbit-serum and this was confirmed by Wu, TenBroeck and Li (1927), a result which suggests that whatever the change produced in the protein molecule may be, it is not so profound as that produced by the introduction into the protein molecule of certain elements or groups. Wu, TenBroeck and Li investigated the immunological properties of pure crystalline egg-albumin which had been denatured by the separate action of heat, alcohol, 0·05 N acid and 0·05 N alkali. They found that denaturation, however effected, markedly reduced antigenic activity, and concluded that when egg-albumin undergoes denaturation the original specificity is destroyed and a new specificity is created, and that the substances produced by the action of heat, alcohol, acid and alkali are closely related immunologically.

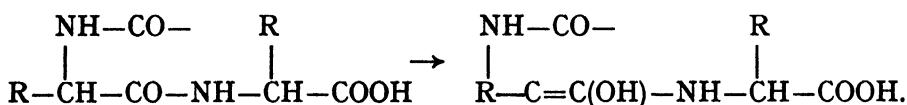
The effect of alcohol on the antigenic properties of proteins varies with the nature of the protein, the concentration of the alcohol, the time of exposure and the temperature. Egg-albumin is easily denatured and coagulated by alcohol, and Wells (1908) showed that alcohol-coagulated egg-albumin failed to produce symptoms of anaphylaxis in sensitized guinea-pigs. Wu, TenBroeck and Li (1927) found that the antigenic activity of crystallized egg-albumin was reduced and that the original specificity was changed after overnight exposure to 40 per cent. alcohol. Serum-albumin is not so easily coagulated as egg-albumin and provided the exposure to alcohol is not prolonged, alcohol-precipitated serum-albumin redissolves and produces shock in sensitized animals (Wells, 1908). Hartley (1925) showed that the proteins of serum after precipitation with alcohol and ether at low temperature retained their antigenic properties unchanged and unimpaired, and Robinson (1928) found that the antisera

prepared by the injection of such lipoid-free proteins exhibited a higher degree of specificity than those prepared against normal unextracted sera. Certain of the proteins of the seeds of cereals are soluble in alcohol and their antigenic properties are unaffected by exposure to this solvent, and proteins with similar properties occur in many species of bacteria. On the other hand, certain kinds of bacteria contain proteins which, like egg- and serum-proteins, are easily denatured and coagulated and rendered antigenically inert by alcohol, and this solvent has been employed in investigating the antigenic structure of bacterial cells.

#### THE EFFECT OF ALKALIS ON ANTIGENIC FUNCTION—RACEMIZATION.

Wells (1909) found that egg-albumin which had been converted into alkali-albumin lost its antigenic properties. The effect of acid was less marked, although antigenic activity was reduced after short exposure to acid. Landsteiner and Barron (1917) also showed that proteins lost their antigenic properties more readily in alkaline than in acid solution, some antigenic activity being retained even after treatment with 5*N* hydrochloric acid for 15 hours at room temperature: antigenic activity was completely lost when the strongly acid mixture was incubated at 37° C. for 24 hours. In contrast, serum exposed to the action of normal alkali for 16 hours at room temperature lost its antigenic properties, and loss of antigenic function after treatment with alkali was also observed by TenBroeck (1914) and Kahn and McNeil (1918).

Kossel and Weiss (1909<sup>1 & 2</sup>, 1910) showed that when certain proteins are incubated in alkaline solution a decline in optical rotation occurs, and this was ascribed to racemization. Dakin (1910) observed a similar change in the alkali salts of optically active hydantoins and ascribed the racemization to tautomeric change of the keto-enol type. Dakin (1912) believed that similar changes would occur in proteins and polypeptides when subjected to the action of alkali according to the following plan:



Thus, of the two amino-acids represented, only the one containing the —CH—CO— group could undergo keto-enol transformation and become racemized. On hydrolysis such racemized proteins should yield amino-acids some of which are optically inactive and some optically active, the latter occupying terminal positions in the polypeptide grouping of the protein molecule. Dakin's method has been of great value, not only in studying the chemical structure of proteins, but also in the study of immunological specificity. Although profound changes occur in the architecture of the protein molecule as a result of the action of alkali, yet racemized proteins retain many essential protein characteristics; they are soluble in water, their solutions coagulate on heating and they contain

the same amino-acids as the proteins from which they are prepared. They are non-toxic, but yield proteoses just as toxic as those prepared from native proteins. They differ from native proteins in being completely resistant to the action of enzymes *in vitro* (this, however, is contested by Lin, Wu and Chen, 1928); when ingested they are excreted unchanged in the faeces and when injected subcutaneously into animals they are excreted unchanged in the urine (Dakin and Dudley, 1913). The first study of the antigenic properties of racemized proteins prepared by Dakin's method was undertaken by TenBroeck (1914), racemized egg-albumin being used. He found that the substance was devoid of all antigenic activity, and later Kahn and McNeil (1918) found that racemized zein, edestin, casein and egg-albumin did not elicit complement-binding antibodies when injected into rabbits. These results appeared to associate the property of antigenicity with parenteral digestion, a view, however, which has been contested by Landsteiner and Barron (1917) who showed that although horse-serum which had been exposed to the action of normal alkali for only 16 hours at room temperature lost its antigenic properties, if such a protein were then converted into xanthoprotein by means of nitric acid, antigenic function was restored, although the precipitin engendered was no longer species-specific. Further, Landsteiner and Jablons (1914) had shown that acetylated horse-serum, though resistant to the action of enzymes *in vitro*, possesses antigenic function. Landsteiner suggests that antigenic activity is dependent upon chemical structure rather than digestibility, and that racemization results in changes whereby the chemical structure necessary for the stimulation of antibodies is lost. It would be of interest to discover whether the products obtained by Dakin's method, which involves a more prolonged (20 days) exposure to alkali at body temperature, would yield antigenic substances when treated in a similar way. Since proteins become non-antigenic after exposure to normal alkali for short periods, and antigenic activity is enormously reduced after exposure to 0·05 N alkali for 24 hours, the conclusion seems warranted that antigenic activity is lost before racemization is complete, and, if this is so, antigenic activity would appear to depend on the occurrence or maintenance of certain chemical groupings within the molecule. These groupings, whatever their nature, appear to be very susceptible to the action of alkali, and Dakin's work has directed attention to an aspect of the chemistry of antigens of exceptional interest.

An important practical outcome of this work is the realization by chemists and immunologists alike that certain apparently simple procedures may produce effects of greater magnitude in the chemical and immunological properties of proteins than has hitherto been generally recognized. Certain proteins are more easily denatured than others, and this is well illustrated in the case of the proteins of bacterial cells. The agents employed for the preparation and purification of proteins—weak acids and alkalis, organic solvents, heat, &c., may cause denaturation

unless great caution is exercised, and may result in the production of substances which, if not entirely inactive, may have undergone profound and possibly undesired alteration in antigenic properties.

#### THE EFFECT OF HYDROLYSIS OF PROTEINS ON ANTIGENIC FUNCTION.

Since antigenic function is so characteristic a property of intact protein molecules, the question as to whether the substances of smaller molecular size which result from the hydrolysis of proteins also possess antigenic function has been the subject of much investigation and discussion.

The final products of complete hydrolysis of proteins, whether effected by enzymes or acids, consist of amino-acids, and the injection of such a mixture invokes no antibody production whatever. The enzymes, pepsin, trypsin and erepsin attack the protein in different ways ; the rate and course of hydrolysis and the nature of the intermediate products formed vary. Pepsin breaks down the large protein molecule into proteoses and peptones, but in a properly conducted peptic digestion amino-acids are not produced. Proteoses, though smaller compounds than proteins, are, nevertheless, substances of high molecular weight which diffuse slowly, though more readily than proteins. Levene, Van Slyke and Birchard (1910, 1911) made a thorough analysis of two members of the group and demonstrated in each the presence of at least 15 amino-acids, including tyrosine and phenylalanine. If antigenic activity is retained by any of the fragments into which the large protein molecule can be split, such activity is more likely to be exhibited by substances of this class than by the less complex compounds obtained by hydrolysis. Trypsin disintegrates the protein molecule in a different way : amino-acids appear at an early stage, and there is a progressive diminution in the original protein remaining undigested. Soluble, coagulable, and possibly unchanged protein, possessing antigenic properties, may remain in tryptic digestion mixtures for a very long time, and in studying the possible antigenic activity of the products of protein hydrolysis the presence of such material is a disturbing factor. Its removal may be difficult, and the processes adopted for its separation may result in loss, or change, of any antigenic activity which the hydrolysis products may possess.

As Wells (1925) has pointed out, a great deal of the work on the immunological properties of the products of protein hydrolysis is of little value because the nature of the changes which occur, and the complex character of the materials used, have not been realized. A further source of error and confusion has arisen from the circumstance that the anaphylaxis reaction has been extensively used in the study of the problem. Proteoses and peptones are toxic substances which, on injection into animals, produce symptoms simulating to some extent those characteristic of true anaphylaxis. These symptoms, however, may be produced in a great variety of circumstances far removed from the rigid conditions essential for the demonstration of true anaphylaxis. That there is an essential difference between proteose intoxication and true

anaphylactic shock is now recognized, but failure to recognize this difference, particularly in the earlier work, resulted in antigenic function being ascribed to substances which merely exhibited inherent toxicity.

The material used for the study of the antigenic properties of the cleavage products of proteins have been Witte's and other peptones, separated fractions of Witte's peptone, digestion mixtures produced by the action of enzymes or other hydrolytic agents on proteins, and separated fractions from such digestion mixtures. In many cases no attempt has been made to study and follow the chemical changes produced, nor even to ascertain whether the material used was free from unchanged or coagulable protein. The results which have been obtained in carefully conducted experiments, in which due regard has been paid to the chemical changes produced by hydrolysis and to the properties of the materials used lead to the conclusion that antigenic function, so characteristic a property of native proteins, is lost when the large protein molecule is broken down into smaller fragments. At what stage in the breakdown of the protein the property is lost is unknown, but all the available evidence supports the view that this occurs at a very early stage and before the substances known as proteoses have been formed.

An important contribution to this subject was made by Wells (1908, 1909), who investigated the action of trypsin on bovine-serum from the chemical and immunological standpoint. Digestion was continued for 16 months and Wells found that the power to sensitize guinea-pigs decreased with the decrease in coagulable protein. Sensitization was effected, however, even after the biuret reaction was negative and coagulable protein could no longer be demonstrated. Wells ascribes the remaining antigenic activity to traces of protein remaining in amounts far too small to be detected by the ordinary chemical tests for proteins. When subjected to the action of pepsin proteins lose their antigenic properties more readily; Wells found, however, that even after 36 days' digestion crystallized egg-albumin still sensitized slightly although coagulable protein could no longer be demonstrated. He also examined the proteoses obtained from the peptic digestion of egg-albumin and the various fractions obtained by the tryptic digestion of the same protein, including peptone and amino-acids, and found them all to be without antigenic function. Fink (1919) examined the products obtained by the hydrolysis of egg-albumin effected by heating for 10 hours in the autoclave at 10 lb. pressure. These were separated and purified by ammonium sulphate fractionation, and Fink found that the fractions precipitated by  $\frac{1}{4}$ ,  $\frac{1}{2}$ ,  $\frac{1}{4}$  and  $\frac{3}{4}$  saturation were devoid of antigenic function, while those precipitated at  $\frac{3}{4}$  and full saturation were antigenic, though less active than native egg-albumin. It is somewhat surprising that any antigenic activity should remain after such drastic treatment, and, moreover, that this should be associated with the fractions requiring the largest concentration of salt for their precipitation, since in such a mixture one would expect the larger molecules to be precipitated with the

lower concentration of salt. The tentative suggestion may be made that the results are possibly due to the presence of ovomucoid in the original raw material, and similar experiments with pure crystalline egg-albumin would yield information on the point.

A few observations have been made which suggest that substances derived from, but having a slightly less complexity than native proteins, possess antigenic activity. Obermayer and Pick (1906) obtained a precipitating serum by the injection of a tryptic digest of coagulated serum which gave a negative biuret reaction and reacted only with the mixture used for its production. Wells (1908, 1909) made a somewhat similar observation in anaphylaxis experiments with the products of the tryptic digestion of ox-serum. He found that at a certain stage of the digestion the mixture reacted much more severely in guinea-pigs sensitized with the mixture than in guinea-pigs sensitized with native serum. The digestion mixture contained, at this stage, a protein hydrolysis product less complex than native protein, but more complex than the proteoses obtained at a later stage. Landsteiner (1925-6, 1927-8) found also that animals sensitized with the peptic digestion products of egg-albumin and egg-globulin were more sensitive to these products than to the native proteins, and was able to separate a fraction which was much more toxic for animals sensitized with the digestion mixture than for those sensitized with native protein.

The changes which occur in proteins during the very early stages of hydrolysis, and under the influence of feeble chemical and physical agencies cautiously applied, raise chemical questions of the greatest interest to immunologists at the present time. From the physical and chemical point of view, the changes so produced in the molecule are, comparatively speaking, slight ; from the immunological point of view they are, in a sense, maximal, since they are accompanied by the alteration, diminution or complete disappearance of the characteristic biological property of antigenicity. By associating immunological methods with physical and chemical methods it is possible that our knowledge of this phase of protein chemistry, and, *pari passu*, of closely related problems in immunology, may be extended. The work of Wasteneys and Borsook (1924-5), for example, who have effected the synthesis of protein from the products of peptic hydrolysis and have made a close study of the conditions governing the reaction, may have interesting applications and developments in immunology, since Gay and Robertson (1912), Landsteiner (1918) and others, have synthesized antigenic substances from the products of protein hydrolysis.

#### CHEMICAL STRUCTURE AND SPECIFICITY.

It is not known upon what property of proteins antigenic function depends ; there is, however, a large and convincing amount of evidence in support of the view that immunological specificity is closely related to chemical composition. For the experimental foundations upon which this

view is based, and even more for the marshalling of the facts in its support, immunologists are indebted to Pick and Silberstein (1928), Landsteiner (1927, 1928) and Wells (1925).

The evidence in support of the view that specificity is dependent upon chemical composition is drawn mainly from three somewhat different types of experimental investigations, viz. the study of the chemical composition and antigenic properties of single, purified, naturally occurring proteins; the study of the effect of the deliberate alteration of the chemical composition of naturally occurring proteins on their antigenic properties; and the study of certain naturally occurring complex antigens in which specificity is determined by the occurrence in the antigen of groupings which are themselves non-protein, and, in the strict use of the word, non-antigenic.

#### *The Chemical and Immunological Relationships of Some Naturally Occurring Proteins.*

The close relationship between chemical composition and specificity became strikingly evident when isolated proteins, purified as far as possible by the methods available, were used in place of such complex mixtures as serum, milk, tissue extracts, bacteria and other cells.

*The proteins of seeds.* The chemical and immunological study of seed proteins has been pursued by Wells and his co-workers during the past twenty years. Wells and Osborne (1911) showed that the legumin from peas, vetch, lentil and horse-bean, which reveal no detectable differences in chemical composition, behave like identical proteins when studied by the anaphylaxis reaction. The gliadin from wheat, apart from a slightly lower specific rotation, is apparently chemically identical with the gliadin from rye, and the two proteins are immunologically indistinguishable. Jones and Gersdorff (1923) could find no difference in the chemical and physical properties of the globulins from the seeds of cantaloup and squash, and Wells showed that the proteins could not be distinguished by anaphylaxis tests. Hordein from barley is closely related to gliadin, and immunologically the two proteins are similar yet distinguishable. Wheat contains another protein, glutenin, which differs from gliadin in composition and properties; immunologically it is related to, but distinct from gliadin and quite dissimilar from hordein. Later (1915) Wells and Osborne showed that the so-called proteoses from seeds, chemically distinct from the other seed proteins, are also immunologically different, and, moreover, the proteoses from different seeds are distinct from one another. Lewis and Wells (1925), in a chemical and immunological study of the alcohol-soluble proteins of two classes of seeds—a 'wheat' group and a 'corn' group—and Wells, Lewis and Jones (1927), in a similar study of the globulins from a number of leguminous seeds, demonstrated immunological similarity between chemically similar proteins and immunological disparity between proteins which are chemically dissimilar.

As a result of their extended observations Wells and Osborne conclude that since chemically similar proteins from seeds of different genera react anaphylactically with one another, while chemically dissimilar proteins from the same seed in many cases fail to do so, the specificity of the anaphylaxis reaction depends upon the chemical structure of the protein molecule rather than upon biological origin. They also suggest that specificity is dependent not upon the protein molecule as a whole, but upon certain groups or radicles and that two or more such groups may occur in one and the same protein. The results obtained with gliadin, hordein and glutenin might then be explained by the occurrence in gliadin and hordein of common reactive groups, and of reactive groups common to gliadin and glutenin which are absent from hordein.

*The proteins of eggs.* Wells (1911) showed that ovomucoid, egg-albumin, egg-globulin and vitellin, which differ in their chemical composition (Osborne and Campbell, 1900; Plimmer and Rosedale, 1925), are distinct and separate antigens; and Hektoen and Cole (1927, 1928) separated five proteins from egg-white and demonstrated antigenic distinction by means of precipitating antisera; the latter authors showed a close immunological relationship between the non-crystallizable conalbumin of hens' eggs and the albumin of fowl-serum. Lewis and Wells (1927) found that avian mucoids are closely related but distinguishable, and that serum mucoids, which could be distinguished from one another, are but little related to the ovomucoids.

Evidence that immunological specificity is dependent, not only upon the amino-acid make-up of proteins, but upon the stereochemical structure of the protein molecule, is furnished by the work of Dakin and Dale (1919), who showed that the crystalline albumin from the eggs of the hen and the duck are distinct antigens; and although by the ordinary methods of analysis no difference in chemical composition could be detected in the two albumins, yet by the application of Dakin's racemization method a distinct difference in structure was revealed; the optical properties of eight amino-acids were identical, but in the case of three others—histidine, leucine and aspartic acid—definite differences in optical activity were observed.

*The proteins of blood.* While albumin and globulin from serum differ markedly in their chemical composition (Abderhalden, 1903, 1905; Hartley, 1914, 1915) euglobulin and pseudo-globulin are chemically very closely related (Obermayer and Willheim, 1911, 1913; Hartley, 1914; Dudley and Woodman, 1918; Woodman, 1921). Dale and Hartley (1916), by means of the anaphylaxis reaction, demonstrated a clear distinction between albumin and globulin; a rigidly specific sensitiveness to euglobulin occurred comparatively frequently while pseudo-globulin sensitized to itself and euglobulin, although the reaction to pseudo-globulin was always definitely superior. Doerr and Berger (1922<sup>1</sup>) confirmed these results, and also showed that the serum-albumin could be separated into two immunologically distinct proteins; chemical evidence of the

occurrence of two different proteins in the albumin fraction has been provided by Obermayer and Willheim (1911, 1913). Doerr and Berger (1922<sup>a</sup>) also showed that the euglobulin and albumin are species-specific, and Hektoen and Welker (1924), by means of precipitin tests, showed that the euglobulin, pseudo-globulin and albumin from ox-, dog-, horse- and human-serum are distinct species-specific antigens. The ordinary methods of analysis of the corresponding proteins from different species shows no clear differences in composition; these methods, however, give no information of the stereochemistry of the proteins, and examination of the proteins from different species by Dakin's method should yield interesting results and show to what extent the species-specificity of naturally occurring proteins is dependent upon chemical structure. Fibrinogen is immunologically distinct from the other proteins of serum, but Hektoen and Welker (1927) have shown that mammalian fibrinogens do not exhibit the same degree of species-specificity as the other proteins of serum. Avian fibrinogens also showed a similar lack of species-specificity, but they are immunologically distinct from mammalian fibrinogens. Evidence of differences in chemical composition of the haemoglobin from different species has been furnished by Reichert and Brown (1909) and Landsteiner and Heidelberger (1923), and immunological differentiation between the haemoglobins of unrelated species and immunological similarity between the haemoglobins of closely related species has been shown by Hektoen and Schulhof (1923), Landsteiner and Heidelberger (1923) and Higashi (1922).

Each protein in serum exhibits two kinds of specificity, one determining species-specificity and the other differentiating the protein from the other proteins of the same species. As in the case of the seed proteins the immunological behaviour of the individual proteins of serum may be ascribed to the presence of two different antigenic groups. At present, one can only speculate as to the nature of these groups. Obermayer and Pick (1906) consider that the aromatic groups are of special importance, while Rimington (1929) has recently drawn attention to the possible significance of carbohydrate groupings.

*The proteins of milk.* The chemical and immunological study of the proteins of milk has not only furnished further evidence of the close relationship between specificity and chemical composition, but has helped to explain some of the contradictory results obtained in the immunological reactions of milk. Crowther and Raistrick (1916) analysed the proteins occurring in the colostrum, and later in the milk of two cows. They showed that caseinogen, lacto-globulin and lactalbumin are sharply differentiated, distinct proteins and have respectively the same composition whether derived from colostrum or milk. They were able to separate the globulin from colostrum into euglobulin and pseudo-globulin, and their analyses showed that the composition of the two proteins was identical, a result which was confirmed later by Dudley and Woodman (1918). Further, the analyses of Crowther and Raistrick for the milk and colostrum globulin were so closely in agreement with those obtained by

## 236 EFFECT OF PHYSICAL AND CHEMICAL AGENCIES ON

Hartley for ox-serum-globulin as to suggest that lacto-globulin is closely related to, if indeed not identical with, serum-globulin. Lactalbumin, however, is essentially different in composition from ox-serum-albumin.

Wells and Osborne (1921) studied these three proteins from milk as well as a fourth, an alcohol-soluble protein prepared by Osborne and Wakeman (1918), the properties and chemical composition of which sharply differentiated it from the other proteins of milk. They showed that these four chemically distinct fractions are also immunologically distinct, and further, that only the lacto-globulin sensitized to ox-serum or reacted in animals sensitized with ox-serum, in striking confirmation of Crowther and Raistrick's observation that the globulins of milk and serum are indistinguishable chemically, while lactalbumin is chemically different from serum-albumin.

It has been frequently observed that caseinogens do not exhibit rigid specificity in immunity reactions. The physical and chemical properties of the caseinogens from different sources are very similar ; the elementary analyses are nearly identical and the caseinogens from cows' and goats' milk yield similar amounts of the same amino-acids on hydrolysis—chemical findings which might be held to explain the apparent lack of immunological specificity. But Dudley and Woodman (1915), by the application of Dakin's method, have demonstrated a difference in the intramolecular structure of cow and sheep caseinogen. A reinvestigation of carefully purified preparations of different caseinogens by more refined immunological methods might show, as in the case of the albumins from hen's and duck's eggs, that the discrepancy between the chemical and immunological results is more apparent than real.

The study of the proteins of blood, milk and eggs has shown that separate distinct antigens occur in the same species of animal, and antigenic distinction has been shown to be associated with demonstrable differences in chemical composition. If immunological specificity is dependent upon chemical composition, it follows that proteins from different species, which, on examination by chemical and physical methods, appear to be identical, should be immunologically indistinguishable or at least exhibit a very close and intimate immunological relationship. Evidence of the existence of this relationship has been furnished by the chemical and immunological study of the proteins of seeds, and Mörner (1894) isolated  $\alpha$  and  $\beta$  crystallins from the lens of the ox, and showed that these differed in their chemical properties ; Hektoen and Schulhof (1924) showed that these are immunologically distinct from one another, but the  $\alpha$  and  $\beta$  crystallins respectively seem to be identical in different species of mammalian lenses, i.e. the lens of different species always contain the same two chemically and antigenically distinct proteins. These lens proteins therefore exhibit an organ-specificity distinct from species-specificity. Comparative chemical studies of the  $\alpha$  and  $\beta$  crystallins from the lens of different species do not appear to have been undertaken.

*The Effect of the Modification of the Chemical Structure of Proteins on Specificity.*

In 1906 Obermayer and Pick showed that when proteins are treated with iodine, nitric acid or nitrous acid substances are obtained which, though still active antigens, have lost their original species-specificity and have acquired an entirely new specificity. Iodized ox-serum, for example, yielded a precipitin which reacted not only with iodized ox-serum, but with iodized serum from other animal species and with iodized egg-albumin and iodized plant proteins. Further, these modified proteins were specific in respect of the introduced element or group : the precipitins for iodized protein do not react with nitro-proteins and vice versa. The profound effect on specificity is shown by the fact that nitrated rabbit-serum, when injected into rabbits, behaves like a foreign protein ; it engenders a precipitin which reacts with all other nitrated serums, but not with serums modified by the introduction of groups other than the nitro-group. Obermayer and Pick consider that the specificity of a protein antigen is associated in part with the physical properties and in part with the chemical structure of the molecule. The former is easily altered by heat, acids, alkalis, toluol, chloroform, &c., and as a result the specificity of the antigen is altered but species-specificity is retained. The other specificity is associated with the chemical structure of the protein and can only be altered by more drastic chemical treatment, such as the introduction into the molecule of iodine or nitro-groups. Since these groups enter the benzene nucleus, Obermayer and Pick consider that the aromatic amino-acids are of paramount importance in determining species-specificity. Many of these important results have been confirmed by later workers, and in a recent careful reinvestigation Wormall has shown that, in the case of halogenated proteins at least, the altered specificity is related to the 3 : 5 dihalogenated tyrosine grouping of the protein molecule.

Landsteiner and his co-workers have shown, however, that species-specificity is just as markedly affected by esterification, acetylation or methylation as by halogenation or nitration (Landsteiner and Prášek, 1914 ; Landsteiner and Jablons, 1914 ; Landsteiner and Lampl, 1917<sup>1 & 2</sup> ; Landsteiner, 1917). The importance of these observations resides in the fact that the changes produced in the protein molecule occur, not in the aromatic nucleus, but in the carboxyl-, hydroxyl- and amino-groupings and such modification in chemical structure results in the loss of species-specificity, and the creation of a new specificity associated with the introduced groupings.

Later, Landsteiner and Lampl (1917<sup>3</sup>) found in the work of Pauly (1915) on the reaction between diazo-compounds and proteins a general method for preparing a long series of 'azo-proteins' particularly suitable for the study of the relationship between chemical structure and specificity. The advantage of this method is that it is possible to attach to a protein

chemical groups of known composition, and thus by means of the precipitin reaction the effect on specificity of position isomerism, of acid groupings of different types, of halogen, nitro- and methyl-groups, and of closely related yet chemically distinct combinations can be investigated. Precipitating antisera were prepared by the injection of these azo-proteins into rabbits, and, in order to avoid species-specific reactions, the antigens used in the precipitin tests were prepared from fowl-serum and occasionally from edestin. It was concluded from these experiments that specificity is determined by the chemical configuration of a relatively small part of the antigen. A few of the antisera were strictly specific; more of them exhibited cross reactions simulating to some extent the group reactions observed in other serological reactions, and when these occurred it was found that such sera reacted with antigens which contained groupings which were isomeric with, or very similar in chemical conformation to, the groupings of the homologous antigen. Thus cross reactions occurred between closely related antigens containing a carboxyl-group; immune sera prepared against antigens containing a sulphonic acid-group showed cross reactions with other antigens also containing this group, while one immune serum prepared against a phenyl arsenic acid azo-protein reacted with six other antigens containing arsenic acid, but did not react at all with other antigens. The relative positions of the acid-groups and the diazo-groups in the azo-protein antigen had an important influence on the scope of the reaction of the immune serum produced. The effect of methyl-groups varied but generally was not marked, and the effect of substituted halogens was less than the introduction of an acid-group. The relative positions of elements and groups undoubtedly play an important part, but the actual chemical nature of a small group or side chain may be equally important. The particularly important part played by acid-groups has been demonstrated by the later work of Landsteiner and van der Scheer (1927).

In later work, Landsteiner (1918) prepared a long series of azo-proteins in which metanilic acid and p-arsanilic acid respectively were combined with proteins of widely different origin—rabbit-serum, lens substance, casein, plant proteins, &c. On being tested with antisera prepared by the injection of metanilic acid azo-protein and p-arsanilic acid azo-protein respectively, it was found that each of the antisera reacted with practically all the various azo-protein antigens containing the same azo-component. The results support the view that specificity is determined by a relatively small part of the protein molecule. The function of the protein is to incite antibody production—a property associated with large molecular size and colloidal properties; specificity is determined by small, perhaps by single, groups.

Landsteiner and van der Scheer (1928, 1929) have shown that in addition to chemical composition and the relative position of reacting groups, steric isomerism is also concerned in the determination of specificity: the dextro and laevo forms of phenyl (p-amino-benzoyl-amino)

acetic acid, and dextro-, laevo-, and meso-tartaric acids, when conjugated to form azo-proteins, yield specific antisera on injection into rabbits.

Landsteiner (1920) also showed that although the various organic substances of known composition which he had coupled to various types of proteins possessed no true antigenic function when free and uncombined, yet they could combine with the specific antibodies which they, by virtue of their association with proteins, had produced in inoculated animals. Thus, for example, if metanilic acid is first added to a precipitating serum prepared by the injection of metanilic acid azo-protein, no precipitation occurs when the homologous antigen—metanilic acid azo-protein—is subsequently added. Further, the reaction is specific : similar inhibition of precipitation is exhibited only by closely related chemical substances and specific precipitation proceeds unhindered in the presence of chemically unrelated substances.

It is unfortunate that the term 'antigen' has been used indiscriminately to describe substances which, on injection into animals, give rise to the production of specific antibodies as well as to substances which, though without this property, nevertheless react specifically with immune sera *in vitro*. To the former group the term antigen is correctly applied and to this group it should be restricted. To the latter group Landsteiner has proposed the term 'haptens', by which is meant substances which are unable to incite antibody production, but have the property of reacting with antibodies : to this group belong the substances of known chemical composition referred to above, similar substances mentioned later, and certain fractions of complex bacterial antigens.

#### *Chemical and Immunological Relationships of Some Naturally Occurring Complex Antigens.*

The results of the studies of artificially modified proteins suggest that while antigenic function is dependent upon the protein molecule as a whole, specificity is determined by chemical structure and may be dependent upon a very small part of the large protein molecule. It is right to enquire whether the study of naturally occurring antigens provides any evidence in support of this view. The results of research in recent years have revealed two classes of naturally occurring antigens, one in certain animal organs and the other in many species of bacteria, the behaviour and properties of which may be explained on the assumption that they owe their antigenic function to a protein portion, which constitutes probably a relatively large part of the complex antigen, and their specificity to a smaller non-protein fraction. The one class is the Forssman antigen (see Chapter IX), the other includes the bacterial polysaccharides.

*The antigenic structure of bacteria—Bacterial polysaccharides.* In 1917 Döchez and Avery demonstrated in culture filtrates of the different types of the pneumococcus substances which reacted specifically with homologous immune sera. These soluble, type-specific substances were also found in the blood and urine of animals inoculated with the pneumococcus, and

in the blood and urine of human beings suffering from pneumonia. The soluble specific substances resisted boiling and the action of trypsin : they were precipitated from their solution by alcohol, acetone, and ether, and did not dialyse.

In 1923 Zinsser and Parker, in continuation of some earlier work by Zinsser on the tuberculin reaction (1921) prepared from cultures of pneumococcus, staphylococcus, typhoid, influenza and the tubercle bacillus, alcohol-insoluble substances which gave none of the usual reactions for protein. These 'residue antigens' were markedly specific in that they gave the precipitin and complement-binding reaction with homologous antisera, although none of them incited antibody production on injection into animals. A striking property of the residue antigens was their stability. Boiling for 1 hour at a reaction of pH 5 to 6 failed to destroy their specific properties, and some withstood autoclaving for 1 hour at 3 to 4 atmospheres : they were more sensitive to the action of alkalis. Zinsser and Parker consider that the residue antigens, acid- and heat-resistant and almost certainly protein-free, are analogous to the pneumococcus substances described by Dochez and Avery.

Heidelberger and Avery (1923) described the preparation of a substance from 8-day cultures of Type II pneumococcus which, though incapable of inciting antibody production, reacted specifically in dilutions of 1 in 3 million with Type II pneumococcus immune serum. Subsequently (1924), by suitable modifications of the original method, similar substances equally specific and equally active were obtained from Type I and Type III pneumococcus cultures. Improvements in technique (Heidelberger, Goebel and Avery, 1925<sup>2</sup>) yielded these 'soluble specific substances', as they have come to be called, if not absolutely pure, at least so free from extraneous foreign substances as to leave little doubt as to their importance in the determination of type-specificity.

The study of the properties and chemical composition of the purest preparations of the three soluble specific substances has shown that, although they possess common features, they are separate and distinct chemical substances. They are all complex polysaccharides which, unlike other known substances of similar composition, give no colour reaction with iodine and resist the action of ordinary amylases. They contain no sulphur and no phosphorus. The solutions of these substances do not give a positive biuret reaction, but all give a positive Molisch reaction in very high dilutions. They do not reduce Fehling's solution in the cold, but on hydrolysis with acids reducing sugars are formed, and as these appear in the hydrolysate the specific activity declines and finally disappears.

The three substances show well-marked differences in properties and composition. The soluble specific substance from Type II pneumococcus is a nitrogen-free, weakly acidic polysaccharide having a specific optical rotation of +74° and yielding glucose on hydrolysis. The substance from Type III pneumococcus is also a nitrogen-free polysaccharide ; it is laevorotary (-33°), but in contrast with Type II substance yields little

glucose on hydrolysis (see also Heidelberger and Goebel, 1926, 1927). The substance from Type I pneumococcus is quite different from the other two ; it contains 5 per cent. of nitrogen which is apparently an integral part of the molecule. The substance is strongly dextrorotary (+300°) and yields reducing sugars on hydrolysis ; these have not yet been fully investigated, but they differ from those obtained from the Type II and Type III polysaccharides and consist probably of galacturonic acid and an amino-derivative of a sugar.

In contrast with the bacterial polysaccharides, which are in the highest degree type-specific, Avery and Heidelberger (1923) found that the bacterial protein from the pneumococcus possessed true antigenic function, but was species-specific and not type-specific. Avery and Morgan (1925) demonstrated the distinctive characteristics of the bacterial proteins and the specific polysaccharides. The latter are unable to stimulate antibody production, but react specifically with homologous antipneumococcus serum ; they do not react, however, with antisera prepared against pneumococcus protein, and antiprotein serum does not agglutinate type-specific strains of pneumococcus. On the other hand, pneumococcus protein antisera precipitate pneumococcus protein from all types of the organism. Avery and Neill (1925) showed that while intact pneumococci yield antisera which agglutinate organisms of the homologous type and precipitate the polysaccharide obtained from the homologous type of the organism, the antiserum prepared against solutions of pneumococci, though containing both protein and polysaccharide but free of all formed elements, reacted with the bacterial protein of the pneumococcus, irrespective of the type, but did not react either with the homologous intact cells or the specific polysaccharides. Avery and Heidelberger (1925), in reviewing these results, point out that the protein and carbohydrate exist in the cells to form an antigenic complex which differs immunologically from either constituent separately. Type-specificity is dependent not only on chemical and antigenic differences of the protein and carbohydrate, but is also related to the structural character and morphological integrity of the cell. How the protein and carbohydrate are combined within the cell is unknown, but it is clear that the compound of protein and carbohydrate is the essential antigen and the one responsible for type-specificity. They point out, further, that the pneumococcus is an encapsulated organism, and there are grounds for the belief that the ectoplasmic layer is composed of carbohydrate material identical with the soluble specific substance, while the somatic substance consists largely of protein which is species- but not type-specific. The situation of the soluble specific substances at the periphery of the cell may thus account for the facility with which the cell reacts in immunity reactions and for specificity. Avery and Heidelberger point out that the confusing cross reactions obtained with antisera supposed to be type-specific find a ready explanation when the nature of the antigen providing the antiserum is considered.

## 242 EFFECT OF PHYSICAL AND CHEMICAL AGENCIES ON

The work of Avery and his colleagues has led to the chemical investigations of the antigens occurring in other bacterial species. Toenniesson (1921) prepared a polysaccharide from a strain of Friedländer's bacillus which, on hydrolysis with acids, yielded a sugar which he believed to be galactose. Mueller, Smith and Litarczek (1924-5) obtained 3 gm. of this material; it contained 1·3 per cent. of nitrogen, some phosphorus but no sulphur, and yielded reducing sugars on hydrolysis. It reacted in high dilution with specific antiserum. Heidelberger, Goebel and Avery (1925<sup>1</sup>), by application of methods essentially similar to those employed in the study of the pneumococcus, isolated the soluble specific substances from Type B Friedländer's bacillus (Julianelle's classification) as a nitrogen-free polysaccharide having a specific rotation of +100°, and demonstrated glucose in the products of acid hydrolysis. In chemical properties the substance resembled closely the soluble specific substance from Type II pneumococcus. These authors also investigated (1925<sup>3</sup>) the immunological relationships of these closely related yet chemically distinct polysaccharides, and demonstrated a close immunological relationship: the substance from Type B Friedländer's bacillus precipitated a specific antiserum and Type II antipneumococcus serum equally well at dilutions of 1 in 2 million, but did not react at all with Type I and Type III pneumococcus antisera. Other immunity reactions demonstrated a close relationship between the soluble specific substances of the two organisms and between the organisms themselves, which is presumably dependent upon a close chemical relationship. Nevertheless, small differences in immunological behaviour, revealed by absorption tests, do exist and this again may be related to the small chemical differences between the soluble specific substances. The authors suggest that both substances contain the same, or a closely similar, configuration of atoms within the complex molecule which determines the close immunological similarity of the two substances. Type II pneumococcus and Type B Friedländer's bacillus provide an example of heterogenetic specificity among bacteria.

Goebel and Avery (1927) prepared the soluble specific substances from Type A and Type C Friedländer's bacillus. They were obtained as nitrogen-free polysaccharides and shown to be chemically distinct and highly specific. The substance from Type A was laevorotary (-100°), that from Type C was dextrorotary (+100°). Both are strong acids, and are non-reducing but yield reducing substances on hydrolysis. The composition of the substance from Type A has been investigated by Goebel (1927), who found that this substance and the polysaccharide from Type III pneumococcus yielded closely related substances. They differ, however, probably in spatial configuration, and immunologically the two polysaccharides are unrelated. The two polysaccharides from Type B and Type C Friedländer's bacillus are also very similar in their properties, although they show certain differences and immunologically they are quite distinct and their antigenic individuality may be due to differences in intramolecular structure.

Mueller and Tomcsik (1924), by a modification of Zinsser's original method, prepared a complex carbohydrate from yeast, which, though incapable of inciting antibody formation, reacted specifically with antisera prepared by the injection of intact yeast-cells. The purest preparations contained small amounts of nitrogen and phosphorus; whether these elements occurred as impurities or combined in the molecule was not decided, but immunization experiments showed that the nitrogen present was not in the form of protein.

Laidlaw and Dudley (1925) obtained from tubercle bacilli (human type) a soluble specific substance which failed to produce antibodies, but gave specific precipitation in a dilution of 1 in 6·4 millions with tubercle antiserum. The substance gave no colour reaction with iodine, and did not reduce alkaline copper solutions. Reducing substances were produced on acid hydrolysis, and although the presence of glucose could not be demonstrated, pentoses were shown to be present to the extent of about 20 per cent. Mueller (1926), starting from old tuberculin and by methods essentially different from those of Laidlaw and Dudley, obtained a substance with very similar properties. These substances are unrelated to tuberculin since large doses produced no reaction in tuberculous guinea-pigs.

Tomcsik (1926-7) isolated from *B. lactis aerogenes* a specific substance which contained 0·9 per cent. of nitrogen even after repeated purification. The substance yielded reducing sugars on hydrolysis and reacted in high dilution with homologous antisera. Similar substances were obtained from other strains and from encapsulated *B. coli*, and these also showed a strict individual specificity.

Landsteiner and Levine (1927) obtained a carbohydrate-like specific substance from *V. cholerae*, and Furth and Landsteiner (1928), in a study of the antigenic structure of organisms of the typhoid group, demonstrated the presence in *B. typhosus*, *B. paratyphosus B.*, *B. enteritidis* and *B. proteus* of substances almost free from protein, rich in carbohydrate-material and yielding reducing sugars on hydrolysis: these substances, though incapable of inciting antibody formation, reacted specifically with homologous antisera.

White (1927, 1928, 1929) demonstrated the presence of specific carbohydrate substances in smooth forms of organisms of the *Salmonella* group and their absence in cultures of the rough variants.

Lancefield (1925, 1928<sup>1</sup>) has shown that the streptococcus antigen contains a protein which is a true antigen in that it incites antibodies on injection. The antisera prepared against this protein, however, show a wide range of reaction, group relationship being demonstrated with the bacterial protein from related Gram-positive cocci. The presence of a polysaccharide was also demonstrated; it was devoid of antigenic function, but reacted in high dilution with antistreptococcus serum. In contrast with the pneumococcus group the polysaccharide was species-specific, but not type-specific. There is also present a type-specific fraction which, according to Lancefield, is undoubtedly a protein, but up to the present

## 244 EFFECT OF PHYSICAL AND CHEMICAL AGENCIES ON

all attempts to produce antibodies to this type-specific fraction have failed. Animals injected with intact streptococci produce antisera which react with the non-specific protein, the species-specific polysaccharide and the type-specific protein, but the antiserum produced by cell-free filtrates or autolysates reacts only with the non-specific protein.

An interesting observation has been made by Heidelberger, Avery and Goebel (1929), who, in view of the wide distribution of bacterial polysaccharides and the part these play in immunological relationships, examined a number of gums of plant origin and found that occasional samples of gum arabic precipitated Type II and Type III antipneumococcus serum in dilutions of 1 : 25,000, and did not react with Type I or normal serum. On partial acid hydrolysis the gum yielded a carbohydrate, comparable in its precipitating activity for Type II and Type III anti-serum with the soluble specific substance of the organisms. About half of the original gum was recovered on hydrolysis, but the precipitating activity of the product was increased 100- to 150-fold, indicating that the reacting substance does not exist free in the gum, but is formed from it. On hydrolysis, the substance yielded galactose and two or more complex sugar acids, one of which resembled to some extent the disaccharide acids obtained from Type III pneumococcus and Type A Friedländer's bacillus. The two latter, however, are composed of glycuronic acid and glucose, while Heidelberger and Kendall (1929) have shown that the disaccharide from gum arabic is composed of glycuronic acid and galactose.

The study of some of these soluble-specific substances by means of the anaphylaxis reaction has yielded interesting results. Tomcsik (1926-7) showed that while the soluble-specific substance from *B. lactis aerogenes* failed to sensitize guinea-pigs, the substance produced typical anaphylactic shock when injected in very small doses into guinea-pigs which had been passively sensitized with *B. lactis aerogenes* immune serum. Similar results were obtained by Tomcsik and Kuretchkin (1928) with the soluble-specific substances from a strain of the pneumobacillus and from yeast. Lancefield (1928<sup>2</sup>) found that neither the species-specific polysaccharide nor the type-specific acid-soluble protein obtained from the streptococcus sensitized guinea-pigs anaphylactically, but each of these substances produced typical anaphylactic shock in guinea-pigs passively sensitized with antistreptococcus serum. In all these cases, however, the polysaccharides used were not entirely free from nitrogen, and although it is very likely, as Avery and Tillett's (1929) work suggests, that the reactions observed were due to the polysaccharide, the possibility that the results were due to other substances, possibly protein in nature, cannot be entirely excluded. Avery and Tillett's (1929) work, however, is not open to this objection. They used the purest preparations of the pneumococcus polysaccharides, and showed that, although these substances failed to sensitize guinea-pigs, they produced rapid, fatal and typical anaphylactic shock in guinea-pigs passively sensitized with the precipitating serum of rabbits immunized with pneumococci of the homologous type ; the reactions were

type-specific. The curious fact emerged from Avery and Tillett's work that there was a complete absence of anaphylactic response in guinea-pigs sensitized with antiserum prepared in horses.

The results obtained by the anaphylaxis reaction thus confirm those obtained by other methods : of equal importance to the actual results obtained is the demonstration that the method can be used, with every prospect of success, for the elucidation of problems concerning the structure of complex bacterial antigens and for the study of the difficult problem of bacterial anaphylaxis.

An interesting development of the recent studies on bacterial polysaccharides, and the investigation of their relation to the whole complex bacterial antigen and to immunological specificity, is seen in the work of Avery and Goebel, who investigated the question whether the antigenic specificity of a protein would be affected by combining with it a relatively simple carbohydrate. Goebel and Avery (1929) succeeded in synthesising the p-amino-phenol glucoside of glucose and galactose and then coupled the diazotized glucosides with horse-serum-globulin and egg-albumin in the same way as Landsteiner prepared his azo-proteins. Four azo-proteins—gluco-globulin, galacto-globulin, gluco-albumin and galacto-albumin—were prepared and these antigens and the immune sera obtained by the injection of rabbits were investigated. They found that when glucose and galactose are coupled to the same protein, the sugar-proteins exhibit distinct immunological specificity, and when the same sugar is coupled to globulin and albumin the sugar-proteins acquire a common immunological specificity. Thus, the newly acquired specificity of the sugar-protein is determined by the chemical constitution of the carbohydrate. Glucose and galactose differ only in their specific rotation and in the disposition of the hydrogen- and hydroxyl-group attached to one asymmetric carbon atom, and yet this difference suffices to determine the specificity of the antigen. These simple carbohydrates exert an effect sufficient to mask the species-specificity of the protein to which they are attached and confer upon the sugar-protein antigen a new specificity dependent upon the chemical structure of the simple sugar radical. Avery and Goebel also found that the p-amino-phenol glucosides, free and uncombined with protein, did not stimulate the production of antibodies, and did not form precipitates when added to the immune serum prepared from the homologous sugar-protein, but the free glucosides inhibited specifically the reaction between sugar-protein and its specific antibody (cf. Landsteiner, 1920). The glucosides, therefore, are carbohydrate haptens ; they are non-antigenic, but specifically reactive, as shown by inhibition tests, with antibodies produced by homologous azo-proteins. These results were confirmed and extended by Tillett, Avery and Goebel (1929) by the anaphylaxis reaction. Guinea-pigs actively sensitized with gluco-globulin, or passively sensitized with anti-gluco-globulin serum, succumbed to injections of gluco-albumin ; similar results were obtained with galacto-protein. The reactions were sharply specific,

and no symptoms of anaphylaxis appeared when heterologous sugar-proteins were injected into sensitized animals. The uncombined glucosides, further, exhibited a specific, but transitory protection.

With the increase of our knowledge of the chemical nature of at least some of the constituents of bacterial cells a somewhat clearer conception of the structure of bacterial antigens has emerged and a beginning has been made in the application of the results so far obtained to the study of other problems in bacteriology and immunology. Thus, Avery and Heidelberger (1925) have pointed out the close relationship between optimal growth conditions provided for the pneumococcus and capsule formation, virulence and type-specificity, characteristics manifested to the full when growth conditions are favourable, while under conditions which are adverse capsular development is feeble or absent, the organisms lack virulence, and type-specificity is lost; elaboration of type-specific polysaccharides proceeds actively when growth conditions are favourable and is absent when growth conditions are adverse. Smith (1927), in a study, of pathogenic *B. coli*, demonstrated a similar close relationship between the production of specific polysaccharides, capsular material, and virulence. Sia (1926) showed that the addition of small amounts of pneumococcus soluble-specific substance conferred on avirulent pneumococci the growth capacity of virulent organisms, and showed also that this action was highly type-specific. Type-specific substances are intimately related to the rough and smooth variants of bacteria: in the pneumococcus group, for example, each of the fixed types occurs in two forms—the smooth, virulent, type-specific variety and the rough, avirulent, non-type-specific form, and these differences in biological properties are related to the presence of specific polysaccharides in the smooth form and their absence in the rough variety (Reimann, 1926). Todd and Lancefield (1928) have described 'matt' and 'glossy' colonies in cultures of haemolytic streptococci. The organisms of the matt colonies are virulent and contain the type-specific substances of the streptococcus, while those from the glossy colonies are avirulent and do not contain the type-specific substance. Further, antisera prepared against the matt form protect mice against infection with homologous virulent organisms, while antisera prepared against the glossy forms do not. In the important field of the standardization of therapeutic sera the results already obtained have found practical application. Friedländer, Sobotka and Banzhaf (1928) have shown that the highly specific polysaccharides from the pneumococcus group may be used for the assay of antipneumococcus serum.

#### REFERENCES.

- ABDERHALDEN, E., 1903, *Hoppe-Seyl. Z.*, **37**, 495; 1905, *ibid.*, **44**, 17.
- ANSON, M. L. & MIRSKY, A. E., 1925, *J. Gen. Physiol.*, **9**, 169.
- AVERY, O. T. & GOEBEL, W. F., 1929, *J. Exp. Med.*, **50**, 533.
- AVERY, O. T. & HEIDELBERGER, M., 1923, *J. Exp. Med.*, **38**, 81; 1925, *ibid.*, **42**, 367.
- AVERY, O. T., HEIDELBERGER, M. & GOEBEL, W. F., 1925, *J. Exp. Med.*, **42**, 709.
- AVERY, O. T. & MORGAN, H. J., 1925, *J. Exp. Med.*, **42**, 347.
- AVERY, O. T. & NEILL, J. M., 1925, *J. Exp. Med.*, **42**, 355.

# THE PROPERTIES OF ANTIGENS AND ANTIBODIES 247

AVERY, O. T. & TILLETT, W. S., 1929, *J. Exp. Med.*, **49**, 251.  
 BEGER, H., 1924, *Zbl. Bakter.*, Abt. I, Orig., **91**, 519.  
 CHICK, H. & MARTIN, C. J., 1910, *J. Physiol.*, **40**, 404; 1911, *ibid.*, **43**, 1; 1912, *ibid.*, **61**, 261.  
 CROWTHER, C. & RAISTRICK, H., 1916, *Biochem. J.*, **10**, 434.  
 DAKIN, H. D., 1910, *Amer. Chem. J.*, **44**, 48; 1912, *J. Biol. Chem.*, **13**, 357.  
 DAKIN, H. D. & DALE, H. H., 1919, *Biochem. J.*, **13**, 248.  
 DAKIN, H. D. & DUDLEY, H. W., 1913, *J. Biol. Chem.*, **15**, 263, 271.  
 DALE, H. H. & HARTLEY, P., 1916, *Biochem. J.*, **10**, 426.  
 DOCHEZ, A. R. & AVERY, O. T., 1917, *J. Exp. Med.*, **26**, 477.  
 DOERR, R. & BERGER, W., 1922<sup>2</sup>, *Z. Hyg. InfektiKv.*, **96**, 191; 1922<sup>2</sup>, *ibid.*, **96**, 258.  
 DUDLEY, H. W. & WOODMAN, H. E., 1915, *Biochem. J.*, **9**, 97; 1918, *ibid.*, **12**, 339.  
 FINK, E. B., 1919, *J. Infect. Dis.*, **25**, 97.  
 FRIEDLÄNDER, M., SOBOTKA, H. & BANZHAF, E. J., 1928, *J. Exp. Med.*, **47**, 79.  
 FUJIWARA, K., 1922, *Deuts. Z. ges. gerichtl. Med.*, Berl., **1**, 562.  
 FURTH, J., 1925, *J. Immunol.*, **10**, 777; 1926, *ibid.*, **11**, 215.  
 FURTH, J. & LANDSTEINER, K., 1928, *J. Exp. Med.*, **47**, 171.  
 GAY, F. P. & ROBERTSON, T. B., 1912<sup>1</sup>, *J. Biol. Chem.*, **12**, 233; 1912<sup>2</sup>, *J. Exp. Med.*, **18**, 479.  
 GOEBEL, W. F., 1927, *J. Biol. Chem.*, **74**, 619.  
 GOEBEL, W. F. & AVERY, O. T., 1927, *J. Exp. Med.*, **46**, 601; 1929, *ibid.*, **50**, 521.  
 HARRIS, L. J., 1923, *Proc. Roy. Soc., B.*, **94**, 426.  
 HARTLEY, P., 1914, *Biochem. J.*, **8**, 541; 1915, *ibid.*, **9**, 269; 1925, *Brit. J. Exp. Path.*, **6**, 180.  
 HEIDELBERGER, M. & AVERY, O. T., 1923, *J. Exp. Med.*, **38**, 73; 1924, *ibid.*, **40**, 301.  
 HEIDELBERGER, M., AVERY, O. T. & GOEBEL, W. F., 1929, *J. Exp. Med.*, **49**, 847.  
 HEIDELBERGER, M. & GOEBEL, W. F., 1926, *J. Biol. Chem.*, **70**, 613; 1927, *ibid.*, **74**, 613.  
 HEIDELBERGER, M., GOEBEL, W. F. & AVERY, O. T., 1925, *J. Exp. Med.*, **42**, 701<sup>1</sup>, 727<sup>2</sup>.  
 HEIDELBERGER, M. & KENDALL, F. E., 1929, *J. Biol. Chem.*, **84**, 639.  
 HEKTOEN, L. & COLE, A. G., 1927, *J. Infect. Dis.*, **40**, 647; 1928, *ibid.*, **42**, 1.  
 HEKTOEN, L. & SCHULHOF, K., 1923, *J. Infect. Dis.*, **33**, 224; 1924, *ibid.*, **34**, 433.  
 HEKTOEN, L. & WELKER, W. H., 1924, *J. Infect. Dis.*, **35**, 295; 1927, *ibid.*, **40**, 706.  
 HIGASHI, S., 1922, *J. Biochem. (Tokyo)*, **2**, 315.  
 HOPKINS, F. G., 1925, *Biochem. J.*, **19**, 787.  
 JONES, D. B. & GERSDORFF, C. E. F., 1923, *J. Biol. Chem.*, **58**, 79.  
 KAHN, R. L. & MCNEIL, A., 1918, *J. Immunol.*, **3**, 277.  
 KOSSEL, A. & WEISS, F., 1909<sup>1</sup>, *Hoppe-Seyl. Z.*, **59**, 492; 1909<sup>2</sup>, *ibid.*, **60**, 311; 1910, *ibid.*, **68**, 165.  
 LAIDLAW, P. P. & DUDLEY, H. W., 1925, *Brit. J. Exp. Path.*, **6**, 197.  
 LANCEFIELD, R. C., 1925, *J. Exp. Med.*, **42**, 377, 397; 1928<sup>1</sup>, *ibid.*, **47**, 91, 469, 481; 1928<sup>2</sup>, *ibid.*, **47**, 843, 857.  
 LANCEFIELD, R. C. & TODD, E. W., 1928, *J. Exp. Med.*, **48**, 769.  
 LANDSTEINER, K., 1917, *Z. ImmunForsch.*, Tl. I, Orig., **26**, 122; 1918, *Biochem. Z.*, **93**, 106; 1920, *ibid.*, **104**, 280; 1924, *J. Exp. Med.*, **39**, 631; 1925-6, *Proc. Soc. Exp. Biol., N.Y.*, **23**, 540; 1927-8, *ibid.*, **25**, 666; 1927, *Klin. Wschr.*, **6**, 103; 1928, *J. Immunol.*, **15**, 589.  
 LANDSTEINER, K. & BARRON, C., 1917, *Z. ImmunForsch.*, Tl. I, Orig., **26**, 142.  
 LANDSTEINER, K. & HEIDELBERGER, M., 1923, *J. Gen. Physiol.*, **6**, 131.  
 LANDSTEINER, K. & JABLONS, B., 1914, *Z. ImmunForsch.*, Tl. I, Orig., **21**, 193.  
 LANDSTEINER, K. & LAMPL, H., 1917<sup>1</sup>, *Z. ImmunForsch.*, Tl. I, Orig., **26**, 133; 1917<sup>2</sup>, *ibid.*, **26**, 258; 1917<sup>3</sup>, *Biochem. Z.*, **86**, 343.  
 LANDSTEINER, K. & LEVINE, P., 1927, *J. Exp. Med.*, **48**, 213.  
 LANDSTEINER, K. & PRÁŠEK, E., 1914, *Z. ImmunForsch.*, Tl. I, Orig., **20**, 211.  
 LANDSTEINER, K. & VAN DER SCHEER, J., 1927, *J. Exp. Med.*, **45**, 1045; 1928, *ibid.*, **48**, 315; 1929, *ibid.*, **50**, 407.  
 LEVENE, P. A., VAN SLYKE, D. D. & BIRCHARD, F. J., 1910, *J. Biol. Chem.*, **8**, 269; 1911, *ibid.*, **10**, 57.  
 LEWIS, J. H. & WELLS, H. G., 1925, *J. Biol. Chem.*, **68**, 37; 1927, *J. Infect. Dis.*, **40**, 316.

## 248 EFFECT OF PHYSICAL AND CHEMICAL AGENCIES ON

LIN, H. K., WU, H. & CHEN, T. T., 1928, *Chinese J. Physiol.*, **2**, 107, 131.  
 MICHAELIS, L. & DAVIDSOHN, H., 1911, *Biochem. Z.*, **33**, 456.  
 MÖRNER, C. T., 1894, *Hoppe-Seyl. Z.*, **18**, 61.  
 MUELLER, J. H., 1926, *J. Exp. Med.*, **43**, 9.  
 MUELLER, J. H., SMITH, D. E. & LITARCEK, S., 1924-5, *Proc. Soc. Exp. Biol.*, N.Y., **22**, 373.  
 MUELLER, J. H. & TOMCSIK, J., 1924, *J. Exp. Med.*, **40**, 343.  
 NICOLAS, E. & KATRANDJIEFF, K., 1928, *C.R. Soc. Biol.*, Paris, **98**, 1310.  
 OBERMAYER, F. & PICK, E. P., 1902, *Wien. klin. Rdsch.*, **16**, 277; 1906, *Wien. klin. Wschr.*, **19**, 327.  
 OBERMAYER, F. & WILLHEIM, R., 1911, *Biochem. Z.*, **38**, 331; 1913, *ibid.*, **50**, 369.  
 OSBORNE, T. B. & CAMPBELL, G. F., 1900, *J. Amer. Chem. Soc.*, **22**, 422.  
 OSBORNE, T. B. B. & WAKEMAN, A. J., 1918, *J. Biol. Chem.*, **33**, 243.  
 PAULY, H., 1915, *Hoppe-Seyl. Z.*, **94**, 284.  
 PICK, E. P. & SILBERSTEIN, F., 1928, *Handb. d. path. Mikroorg.*, hrsg. Kolle, Kraus u. Uhlenhuth, Jena, **2**, 333.  
 PLIMMER, R. H. A. & ROSEDALE, J. L., 1923, *Biochem. J.*, **19**, 1015.  
 REICHERT, E. T. & BROWN, A. P., 1909, *Pub. Carnegie Instn.*, 116.  
 REIMANN, H. A., 1926, *J. Exp. Med.*, **43**, 107.  
 RIMINGTON, C., 1929, *Biochem. J.*, **23**, 430.  
 ROBINSON, S. H. C., 1928, *Brit. J. Exp. Path.*, **9**, 299.  
 ROSENBERG, R., 1926, *Zbl. Bakt., Abt. I, Orig.*, **98**, 259.  
 SCHMIDT, C. L. A., 1921, *J. Immunol.*, **6**, 281.  
 SCHMIDT, W. A., 1908, *Biochem. Z.*, **14**, 294; 1909, *ibid.*, **24**, 45; 1912, *Z. ImmunForsch.*, Tl. I, Orig., **13**, 166.  
 SIA, R. H. P., 1926, *J. Exp. Med.*, **43**, 633.  
 SMITH, D. E., 1927, *J. Exp. Med.*, **46**, 155.  
 SØRENSEN, M. & S. P. L., 1924, *C.R. Lab. Carlsberg.*, **15**, No. 9.  
 TENBROECK, C., 1914, *J. Biol. Chem.*, **17**, 369.  
 TILLETT, W. S., AVERY, O. T. & GOEBEL, W. F., 1929, *J. Exp. Med.*, **50**, 551.  
 TODD, E. W. & LANCEFIELD, R. C., 1928, *J. Exp. Med.*, **48**, 751.  
 TOENNIESSEN, E., 1921, *Zbl. Bakt., Abt. I, Orig.*, **85**, 225.  
 TOMCSIK, J., 1926-7, *Proc. Soc. Exp. Biol.*, N.Y., **24**, 810, 812.  
 TOMCSIK, J. & KUROTKHIN, J. J., 1928, *J. Exp. Med.*, **47**, 379.  
 WALKER, E., 1925, *Biochem. J.*, **19**, 1082.  
 WASTENEYS, H. & BORSOOK, H., 1924-5, *J. Biol. Chem.*, **62**, 15, 633, 675; 1925, *ibid.*, **63**, 563.  
 WELLS, H. G., 1908, *J. Infect. Dis.*, **5**, 449; 1909, *ibid.*, **6**, 506; 1911, *ibid.*, **9**, 147; 1913, *Z. ImmunForsch.*, Tl. I, Orig., **19**, 599; 1916, *J. Biol. Chem.*, **28**, 11; 1925, *The Chemical Aspects of Immunity*, New York.  
 WELLS, H. G. & LEWIS, J. H., 1924, *J. Biol. Chem.*, **59** (*Proc. Amer. Soc. Biol. Chemists*, iii).  
 WELLS, H. G., LEWIS, J. H. & JONES, D. B., 1927, *J. Infect. Dis.*, **40**, 326.  
 WELLS, H. G. & OSBORNE, T. B., 1911, *J. Infect. Dis.*, **8**, 66; 1915, *ibid.*, **17**, 259; 1921, *ibid.*, **29**, 200.  
 WHITE, P. B., 1927, *J. Path. Bact.*, **30**, 113; 1928, *ibid.*, **31**, 423; 1929, *ibid.*, **32**, 85.  
 WOODMAN, H. E., 1921, *Biochem. J.*, **15**, 187.  
 WORMALL, A., 1930, *J. Exp. Med.*, **51**, 295.  
 WU, H., TENBROECK, C. & LI, C. P., 1927, *Chinese J. Physiol.*, **1**, 277.  
 WU, H. & WU, D. Y., 1925, *J. Biol. Chem.*, **64**, 369.  
 YOUNG, E. G., 1922, *Proc. Roy. Soc. B.*, **93**, 15, 235.  
 ZINSSER, H., 1921, *J. Exp. Med.*, **34**, 495.  
 ZINSSER, H. & OSTENBERG, Z., 1914, *Proc. N.Y. Path. Soc.*, **14**, 78.  
 ZINSSER, H. & PARKER, J. T., 1923, *J. Exp. Med.*, **37**, 275.

### Antibodies.

The chemical nature of antibodies is unknown. Whether the striking differences in the biological properties of the sera of normal and immunized animals are due to alteration in the physical properties of normally occurring constituents or to the presence of chemical substances produced by immunization is still undecided. There are difficulties in explaining certain of the properties of antisera on purely physical grounds, and it is generally assumed that the specific properties are due to material substances. The older view that antitoxic action, precipitation, agglutination, lysis, &c., are due to separate and distinct antibodies is now questioned. Although the antitoxins possess distinctive properties which justify their consideration as a special group, there is evidence that other antibody reactions are due to one and the same thing, and that the different manifestations of antibody properties are dependent upon the conditions under which the reaction takes place: some reactions, for example, require the presence of electrolytes, others the presence of complement. The terms agglutinins, precipitins, lysins, &c., are used for convenience to indicate a particular antibody property rather than the occurrence of separate and distinct substances in the antiserum. But assuming that antibodies are material entities, it is yet undecided whether these are new chemical substances which exist free in the blood, or whether they are so intimately associated with other constituents of the blood as to resist all attempts at their separation, or whether they are normally occurring substances which have become changed or modified by immunization and have thus acquired new and specific biological characteristics.

In view of the striking effects produced by extremely small quantities of antisera, it is easy to understand the optimistic spirit in which certain problems concerning the nature of antibodies and their isolation were approached. It was reasonable to expect that the changes in biological properties which occur in the serum of animals during immunization would be accompanied by changes which could be detected by chemical and physical methods; and remembering the signal triumphs achieved in other fields with equally unpromising material, the immunologist had some grounds for hoping that the chemist would be equal to the task of isolating the substances in antisera which, presumably, confer upon them their remarkable properties.

It is now known that the activity of antisera is associated with an extremely small amount of their total solids. The experimental investigation of antibodies, and of the effect of physical and chemical agencies upon them, has of necessity been pursued in face of the difficulty which arises from the fact that they occur in fluids which contain, in addition and in large quantity, some of the most complex substances known. The results of experiments conducted with such crude material as antiserum, native or partially purified, have shown that antibodies possess many

common properties. They are complex substances of high molecular weight which diffuse very slowly and do not dialyse. They are attacked by enzymes and are invariably associated with the globulin fraction of the serum-proteins. They are susceptible to the influences and reagents which affect proteins, and the weight of evidence is in favour of the view that antibodies are proteins ; nevertheless, their behaviour and properties might be equally well explained on the assumption that they are substances of very complex composition and as yet undetermined nature which are adsorbed upon serum-globulin.

#### THE INFLUENCE OF PHYSICAL AND CHEMICAL AGENCIES ON THE ACTIVITY OF ANTIBODIES.

##### *Desiccation.*

Solutions of antibodies may be dried without loss of specific activity and the final products retain their specific properties apparently indefinitely. The method adopted for the desiccation of antibody solutions varies according to the amounts involved and the purpose for which the final product is required. Martin's (1896) method, in the experience of the writer, is practicable in the laboratory for drying a few litres of antiserum (Hartley, Eagleton and Okell, 1923). The drying of small quantities of antiserum is commonly effected in vacuum desiccators containing dehydrating agents. Desiccation of antisera may give variable results : at times the final dried product may dissolve completely and show full activity, at others the reconstructed serum may be cloudy or partly insoluble and of reduced activity. Experiments (unpublished) have shown that the quality of the sulphuric acid, if this is used, is very important, and it is necessary to avoid the development of an acid atmosphere in the desiccator if perfectly and permanently soluble dry products of full biological activity are to be secured.

The writer's practice has been to use pure sulphuric acid, frequently renewed, in well-made desiccators containing a small manometer, and to place on the tray small beakers containing solid caustic potash. When the product appears dry to naked-eye inspection it is transferred to desiccators containing phosphorus pentoxide and the desiccation completed. The phials of dry antisera are then evacuated or filled with dry nitrogen and sealed. Preparations of antisera and toxins prepared in this way and stored at 0 to 4° C. are perfectly soluble and have retained their full activity for periods varying from 6 years to 1 year (not tested at longer intervals in the latter case). When the amounts of serum are small it is preferable to avoid the use of sulphuric acid altogether and use caustic potash or phosphorus pentoxide as the dehydrating agent throughout ; if sulphuric acid must be used, the time of exposure should be reduced to a minimum, to obviate exposure of the product to the action of acid with consequent denaturation and loss of activity. In the case of certain materials a further advantage is gained by preliminary freezing after the method of Shakell (1909).

Antisera may be reduced to the dry condition by precipitation at low temperatures with absolute alcohol, alcohol-ether mixture, or acetone without loss of activity, and provided the organic solvent is completely removed, the products remain soluble and stable (Hardy and Gardiner, 1910 ; Hartley, 1925). It is inadvisable to grind dried antisera, as this causes part of the material to become insoluble in water or salt solution (Hartley, Eagleton and Okell, 1923).

#### *Heat.*

In the fluid condition the specific activity of antisera tends to diminish. The stability of different antibodies varies, but the rate of decline of activity is similarly affected by exposure to heat ; it is very slow at temperatures of 0 to 4° C., greater at room temperature, and with increasing temperatures the rate of decline increases until at temperatures in the neighbourhood of coagulation it disappears.

The effect of heat on diphtheria antitoxin has been studied more completely than on other antisera. Anderson (1910) and MacConkey (1912) reported that diphtheria antitoxin lost about 6 per cent. of its activity after being stored for a year in the ice-chest, and about 10 per cent. per annum at 15° C. MacConkey also found that antitoxin stored at 36° C. lost half its activity in a year, and Glenny (1913) found that antitoxin stored at 37° C. for 7 years lost over 90 per cent. of its activity. Glenny showed, moreover, that under these conditions the properties of antitoxin are modified. The effect of higher temperature is well illustrated by Madsen and Walbum's (1917) experiments, which showed that at 65° C. diphtheria antitoxin lost 70 per cent. of its activity in 6 hours, at 70° C. over 90 per cent. in 3 hours, and at 74° C. very little activity remained after an hour's heating. Madsen concluded that the destruction of antitoxin by heat proceeds as a bimolecular reaction, the temperature coefficient, like that of the denaturation of proteins, being very high. Antitoxins may, however, be heated to 56° C. for several hours without appreciable loss, and this is of importance in the technical processes adopted for the concentration of antitoxic sera.

Agglutinins vary in their resistance to heat. The agglutinins for plague are said to be destroyed by heating to 56° C., but as a rule heating at this temperature has little effect ; the agglutinating properties of most antisera are destroyed by heating to 70 to 80° C. Streng (1909) and Madsen and Streng (1909) investigated the effect of heating typhoid and *B. coli* agglutinins at temperatures ranging from 65 to 75° C. : in some cases the rate of destruction appeared to conform to a monomolecular, and in others to a bimolecular, reaction. Since many species of bacteria contain more than one antigenic protein, agglutinating sera frequently contain more than one agglutinin. In 1903 Joos showed that the typhoid bacillus contained a heat-labile and a heat-stable antigen which engender specific agglutinins, and these in their turn exhibit different stability towards heating : in the case of this organism the heat-labile antigen

produced the more heat-stable agglutinin. These observations have been extended to the study of the antigenic structure and biological relationships of closely allied bacterial species. Pick (1902), by means of ammonium sulphate fractionation, obtained solutions of typhoid and cholera agglutinins, and found that the former were more resistant to heat than the latter. It is probable that in this, as in other experiments in which very stable agglutinins were described, Pick was working with solutions containing H agglutinins.

Slight variations in the susceptibility of precipitins towards heat have been observed. Heating for short periods to 60° C. has little effect, but most precipitating antisera lose their activity by heating to 68 to 75° C. for half an hour. Bacterial precipitins, according to Pick (1902) and Kraus and von Pirquet (1902), are more susceptible, being destroyed by heating to 60° C. Kraus and Joachim (1904) showed that more than one precipitin may be demonstrated in antisera containing bacterial precipitins, one being relatively heat-stable and withstanding heating to 80° C. and the other being destroyed at 60° C. ; the former is produced by the heat-labile and the latter by the heat-stable precipitinogen.

Jones (1927) has shown that antibody destruction increases progressively with increased temperature. At 65° C. for 20 minutes some destruction of agglutinins, precipitins, haemolysin and red-cell agglutinins occurred : the destruction was more marked at 70° C. At 75° C. agglutinin and precipitin were completely destroyed ; at 80° C. red-cell agglutinin was destroyed, but some activity remained in the haemolysin ; this latter was completely destroyed at 85° C. Some H agglutinin remained undestroyed, even after heating to 90° C. for 20 minutes. Jones considers that actual destruction of antibodies occurs as a result of heat.

Felton and Bailey (1926) investigated the effect of heat on six samples of antipneumococcus serum and two samples of purified antibody solution and found that heating to 56° C. for 30 minutes caused marked reduction in the agglutinating, precipitating and complement-fixing properties of the sera and antibody solutions. The protective antibodies in the serum were unaffected, but in one sample of purified antibody solution they were slightly, and in the other very markedly, reduced.

#### *Light.*

The specific activity of antisera declines slowly on exposure to light. Müller (1898) showed that diphtheria antitoxin was more susceptible to blue and green light than to red and yellow light. Baroni and Jonescu-Mihaiesti (1910) found that antibodies were destroyed rapidly on exposure to ultraviolet light, the lytic antibodies being somewhat more easily destroyed than the agglutinins and antitoxins ; normal serum, after prolonged exposure to ultraviolet light, could no longer be precipitated by a specific antiserum. These results were confirmed by Scott (1911), who also found that precipitins are similarly destroyed and that exposure of normal serum to ultraviolet light renders the proteins incoagulable,

and half saturation with ammonium sulphate leads to practically complete precipitation of the proteins. Heuer (1922) showed that the rate of destruction was dependent on the concentration of protein in solution.

#### *Hydrolysis by Enzymes.*

Pick (1902) found that diphtheria antitoxin lost little activity after five days' digestion with trypsin ; after nine days most of the protein had been digested and about two-thirds of the antitoxin had been destroyed. Pepsin destroys antitoxin more rapidly. Mellanby (1908), Banzhaf (1911) and Berg (1921) obtained similar results. The claims of Pröscher (1902) that a protein-free antitoxin may be obtained by the prolonged digestion of antiserum by trypsin has not been confirmed. Agglutinins and precipitins are slowly destroyed by trypsin and more rapidly by pepsin, and in this respect the antibodies resemble the serum-globulins. Huntoon, Masucci and Hannum (1921), however, have reported that a potent and highly purified preparation of pneumococcus antibody showed no loss of activity after a week's digestion with trypsin.

#### *Alcohol.*

The effect of alcohol on antibodies is closely related to denaturation and coagulation of the serum-proteins. Mellanby (1907) showed that alcohol has a critical temperature for the precipitation of the serum-proteins at which the precipitating power of any percentage of alcohol is at a minimum. Below this temperature the addition of more alcohol precipitates the proteins, in amounts proportional to the alcohol added, and the precipitated proteins are not denatured and redissolve. At higher temperatures the same relations hold, but the alcohol, in addition to precipitating the proteins, also causes denaturation and coagulation. Mellanby's experiments were extended later (1908) to diphtheria antitoxin, and it was shown that the antitoxic value was not affected by alcohol so long as no precipitation occurred, i.e. at low concentrations. With higher concentrations the rate at which antitoxin was destroyed was the same as that at which the proteins were coagulated. He also found that the greater part of the antitoxin was precipitated, with other proteins of the serum, by 28 per cent. alcohol at 2° C., and while the same amount of antitoxin was precipitated at temperatures above the critical point in the latter case some of the antitoxin was lost owing to the coagulating action of the alcohol. Hardy and Gardiner (1910) found that antitoxin was precipitated completely with the proteins when the serum was added to ten volumes of alcohol or acetone at 0° C. Hartley (1925) showed that diphtheria antitoxin, typhoid agglutinating serum, and hæmolytic amboceptor retained their specific properties unchanged after precipitation with alcohol and ether at low temperatures. Diphtheria antitoxin neutralized toxin in exactly the same way as the unextracted serum, although its capacity to produce a precipitate with toxin had disappeared. The extracted agglutinating serum agglutinated *B. typhosus*, and the extracted hæmolytic amboceptor lysed red cells to the same titre as the

unextracted sera. It was also shown that precipitating sera treated in this way, when mixed with a similarly extracted antigen, produced no precipitate, although it was demonstrated that union of antigen and antibody had occurred in the normal way.

*The Effect of other Chemical Substances—Antiseptics and Preservatives.*

Antibodies are destroyed by strong acids and alkalis and by substances which cause denaturation and coagulation of the serum-proteins. The different antibodies vary in their stability towards acids and alkalis in weak concentration. The antitoxins are more susceptible to acids than to alkalis, and Hartley (1914) found that the protective antibodies of antirinderpest serum were similarly affected. Agglutinins are more susceptible to alkalis than to acids; Olitzki (1928) found that the O agglutinins are more susceptible to the action of hydrogen and hydroxyl ions than the H agglutinins. Recent investigations, however, show that certain antibodies, e.g. the pneumococcus protective antibody and purified haemolytic amboceptor, are not destroyed by short exposure to weak acids and alkalis at ordinary temperatures.

Neutral salts precipitate the antibodies from their solutions along with the serum-globulins: suitable concentrations of magnesium, ammonium and sodium sulphates precipitate the antibodies quantitatively and exert no destructive or modifying action on their specific properties. These salts are used for the purification and concentration of therapeutic sera. Antitoxin is soluble in saturated solutions of sodium chloride, and MacConkey (1924<sup>1</sup>) has shown that when preserved under these conditions the titre remains unchanged for years.

The effect of antiseptics is important from the practical aspect of the preservation of antisera. Phenol and cresol, in concentrations of 0·5 and 0·3 (or 0·4) per cent. respectively, are most commonly used for the preservation of therapeutic sera. These are not ideal substances for the purpose, but one of their defects, viz. the tendency to cause protein precipitation on addition to the finished product, has been overcome to a considerable extent by dissolving the phenol or cresol in an equal volume of ether before addition to the serum (Krumwiede and Banzhaf, 1921). Although phenol and cresol in these low concentrations have little effect on antibodies at ice-box temperature destruction is more rapid at ordinary and body temperature, and Glenny, Pope, Waddington and Wallace (1925<sup>1</sup>) showed that heating to 60° C. for an hour in the presence of 0·3 per cent. cresol caused a loss of 10 to 30 per cent. of antitoxin units.

Formaldehyde, even in low concentration, causes gel formation in serum, and Glenny and his colleagues (1925<sup>2</sup>) showed that 0·2 per cent. formaldehyde added to diphtheria antitoxin destroyed more than half the activity in three days, and more than three-quarters when the concentration was raised to 0·5 per cent. Baivy (1926) reports that precipitins and haemo-agglutinins lose their activity completely when exposed to the action of formaldehyde in 0·1 per cent. concentration, while bacterial

agglutinins and haemolysins are destroyed more slowly. Olitzki (1928) found that the O agglutinins are more easily destroyed by formaldehyde than the H agglutinins.

Glycerol is specially useful for the preservation of diagnostic serum and for the preparation of the standard serum solutions used for the biological assay of therapeutic sera. The glycerol is used in 66 per cent. concentration. The writer has found that a preparation of diphtheria antitoxin in this menstruum retained its full activity after being stored at 0 to  $-4^{\circ}\text{C}$ . for 5 years. Bitter (1922) states that the agglutinating and haemolytic properties of antisera remained unchanged for years when preserved in glycerol.

Advantages have been claimed for low concentrations of mercuric chloride (Darányi, 1928) and boric acid (Baker, 1925) as serum preservatives

#### THE DISTRIBUTION OF ANTIBODIES IN ANTISERA.

Problems relating to the nature of antibodies, their distribution in and separation from the antisera in which they occur, are inseparably bound up with the study of the serum-proteins.

Although very diverse views are still held regarding the number, nature, origin and function of the proteins of serum, it is generally agreed that serum contains two main groups of proteins, albumins and globulins, and that the globulins can be subdivided into two fractions—euglobulin and pseudo-globulin. The classification was based primarily upon differences in solubility in water and salt solution. Euglobulin is the least soluble fraction, and, in immunological literature particularly, the term is applied indiscriminately to the proteins precipitated by dialysis, or by dilution and acidification, or by saturation with common salt, or by one-third saturation with ammonium sulphate. Pseudo-globulin is not precipitated by dialysis as ordinarily conducted, and is soluble in saturated salt solution and one-third saturated ammonium sulphate solution, but is precipitated by one-half saturation with ammonium sulphate. Albumin is soluble in half-saturated ammonium sulphate or saturated magnesium sulphate, but is precipitated by full saturation with the former salt. Recent chemical and immunological research suggests that each fraction consists of a mixture of proteins, the chemical and physical properties of which are distinct, though closely related.

Salting-out methods have been widely used in the study of normal and immune sera. Since the publication of Hofmeister's classical work these methods have been subjected to critical examination, and their limitations, as applied to the study of protein solutions, have been repeatedly pointed out. It is now known that a complete separation is never effected by a single precipitation, that the amount of precipitate produced is dependent, not only on the concentration of the neutral salt added, but upon the number and concentration of the different proteins in solution, the temperature, and to a remarkable degree upon the hydrogen-ion concentration. By repeated precipitation under carefully controlled

## 256 EFFECT OF PHYSICAL AND CHEMICAL AGENCIES ON

conditions a more perfect separation may be effected, and chemical examination and the application of immunological methods have shown that albumin and globulin are different chemical substances. The separation of albumins and globulins is relatively simpler than the separation of the two globulins, which are chemically and immunologically very closely related.

Salting-out methods, in spite of their imperfections and limitations, have found useful application in the study of antisera. By their use the distribution of antibodies in immune sera has been investigated, practical methods have been evolved for removing at least some of the proteins of serum which are not associated with antibodies, and, by adopting certain precautions and fixed conditions for working, the methods have been of value in studying the changes which occur in the serum-proteins of animals during infection and immunization.

It has been shown that antibodies are sometimes associated with the euglobulin, and sometimes with the pseudo-globulin fraction of the serum-protein, depending upon the antibody concerned and the species of animal furnishing the antiserum. In horses immunized against diphtheria and tetanus toxins the antitoxins are associated with the pseudo-globulin. Pick (1902) stated that diphtheria antitoxin in the goat is associated with the euglobulin fraction, but Ledingham (1907) and Gibson and Collins (1907) were unable to confirm this observation. In a more detailed study of the serum from three immunized goats Banzhaf and Famulener (1914-15) found that the euglobulin contained about twice as much antitoxin as the pseudo-globulin.

With regard to the distribution of other antibodies the reported results are very contradictory. This is due in large measure to the variable methods which have been used for fractionating the serum-proteins, the limitations of salting-out methods not always being realized. Another possible contributory cause of the variability in the reported results may be, as Ledingham (1907) suggested, that the distribution of antibodies among the serum-globulins may vary at different stages of immunization. Ornstein (1928) has recently obtained evidence in support of this view and has also shown that animals of the same species show individual variation. Pick (1902) states that cholera lysin and cholera agglutinin occur in the euglobulin fraction of the serum of immunized horses and goats, while typhoid agglutinin occurs in the euglobulin fraction of goat-, rabbit- and guinea-pig-serum and in the pseudo-globulin fraction of the serum of immunized horses. Gibson and Collins (1907) were unable to confirm these results. Kapsenberg and Rispens (1927) fractionated the agglutinins in horse- and rabbit-sera by ammonium sulphate and by other methods ; they found agglutinins in both globulin fractions and state that the agglutinin content was proportional to the quantity of globulin in the separated fractions ; no difference was observed between horse- and rabbit-sera. While earlier workers state that precipitins are associated with the euglobulin fraction, Doerr and Hallauer (1926) found that

precipitins and anaphylactic antibodies occurred in both globulin fractions ; they were, however, always associated with the same fraction of the serum-protein, and they consider that there is an essential identity between the two antibodies, which is the usually accepted view. Ruppel (1923) and his associates (1923) state that agglutinins are associated mainly with the euglobulin fraction ; they make the curious observation that while complement-binding antibodies occur in the euglobulin, amoceptors occur in the pseudo-globulin. In view of the variable methods employed and the contradictory results obtained in this field, the validity of many conclusions drawn, particularly those relating to the question of the unity or diversity of antibodies, is questioned. Hartley (1914<sup>1</sup>) showed that the antibodies of antirinderpest serum are associated exclusively with the euglobulin fraction. Of the therapeutic sera used for treatment and prophylaxis, scarlet-fever antitoxin, like diphtheria and tetanus antitoxins, appears to be associated with the more soluble part of the pseudo-globulin, while the active principle of antidysentery serum and certain antibacterial sera is found in the less soluble part. The pneumococcus antibody is also precipitated by relatively low concentrations of ammonium sulphate, and, according to Modern and Wernicke (1929) the protective antibodies of anti-anthrax serum occur in the euglobulin fraction.

#### COMPARISON OF THE CHEMICAL AND PHYSICAL PROPERTIES OF NORMAL SERA AND ANTISERA, AND OF THE GLOBULINS OF THESE SERA.

The invariable association of antibodies with the globulin fraction of immune sera has vested this group of the serum-proteins with a special interest to immunologists. It is natural to inquire whether immune serum, and particularly the globulins of immune sera, exhibit any chemical or physical differences from normal serum or the globulins from normal serum. Except for a slight difference in optical rotation and a rather higher coagulation temperature, Seng (1899) could find no difference between the pseudo-globulins of normal and antitoxic sera. Beljaeff (1903) found no difference in the refractive index or freezing-point of normal and agglutinating or antitoxic sera. Banzhaf, Sugiura and Falk (1917) analysed the pseudo-globulin fraction from diphtheria and tetanus antitoxin and obtained figures which revealed no significant difference from those obtained by Hartley (1914<sup>2</sup>) for the pseudo-globulin from normal horse-serum. Reitstötter (1920) observed that the electrolyte-free globulins from antitoxic sera exert a greater sensitizing action on colloidal ferric hydroxide than the corresponding globulins from normal or antibacterial sera. Du Noüy (1923) found no difference in the refractive index of normal and immune sera, but notes that the fall in surface tension which occurs in diluted serum on standing is from 50 to 100 per cent. greater in immune than in normal serum. Tadokoro and Nakayama (1920) found that the absorption band between the wave-lengths of 2,950 and 2,400 Å. of agglutinating and precipitating sera was from 2 to 10 times greater than that of normal serum. Kirkbride and Murdick (1927) found fairly

## 258 EFFECT OF PHYSICAL AND CHEMICAL AGENCIES ON

constant amounts of inorganic constituents in normal and antitoxic sera with the possible exception of sulphur and calcium, which were at times, but not regularly, increased in the latter.

Normal sera and antisera, and the globulins from these, show little if any difference in their chemical and physical properties. Since it has been shown that diphtheria antitoxin, the pneumococcus protective antibody, typhoid agglutinin and haemolytic amoebocyte constitute an infinitesimal part of the total solids of the sera in which they occur, and since these antibodies if not actually proteins are very similar to proteins, any distinctive chemical or physical properties which they may possess would be difficult to demonstrate in the presence of such overwhelmingly large amounts of similar substances.

### THE INCREASE IN GLOBULIN IN THE SERUM OF IMMUNIZED ANIMALS.

The most striking change which occurs in the serum of animals during and after immunization is increase in the globulin fraction of the serum-proteins. This has been shown most frequently in the serum of horses undergoing immunization against diphtheria toxin. Joachim (1903) found that the increase affected the euglobulin fraction, Ledingham (1907) that an increase occurred in both the globulin fractions, and Gibson and Banzhaf (1910), Reymann (1924) and Kirkbride and Murdick (1927) found that the increase affected chiefly the pseudo-globulin fraction. Langstein and Mayer (1904) found that the globulin content of the serum increased during immunization against a variety of micro-organisms. Hartley (1914<sup>1</sup>) demonstrated a close relationship between the euglobulin fraction of the serum of bovines and reaction towards rinderpest infection and immunization. During an attack of rinderpest ending fatally the absolute and relative amounts of euglobulin decreased; the serum of animals immunized and hyperimmunized against rinderpest, however, showed a large increase in euglobulin, and repeated large bleedings were accompanied by a steady decrease in euglobulin and antibody content of the serum, and when such animals were again hyperimmunized the euglobulin was again increased. The total protein increased in all immunized animals, and this increase was related in all cases to the euglobulin fraction, the albumin and pseudo-globulin being practically unaffected. Hurwitz and Meyer (1916) observed an increase in globulin in the serum of rabbits immunized against living and dead micro-organisms and Doerr and Berger (1921) and Berger (1922) in the serum of animals immunized against foreign proteins.

The question of the relationship of globulin increase to the process of immunization and the occurrence of antibodies has been the subject of much discussion. Thus, while Ledingham (1907) found that a refractory horse, in which the initial globulin content of the serum was high, failed to exhibit the characteristic globulin increase, while another horse which yielded a high-grade antitoxin exhibited a large globulin increase, Gibson and Banzhaf (1910) found that the same changes may occur in the serum

both of refractory horses and of those yielding high-grade antitoxin. They also found that although the greatest globulin content was usually coincident with maximum antitoxin development, the globulin increase tended to precede the increase in antitoxin production ; moreover, when the results for different horses were compared, it was found that globulin increase was independent of antitoxic potency. In recent years the lack of any constant relationship between globulin increase and antibody production has been frequently demonstrated. Glaessner (1905) made the important observation that globulin increase occurred only in those animals which reacted severely to the antigen and he showed that no globulin increase occurred when the dosage was carefully controlled and the animals remained in good condition. Moll (1906) disagreed with Glaessner's views, but Müller (1905), Hurwitz and Whipple (1917), Schmidt and Schmidt (1917), Meyer, Hurwitz and Taussig (1918) succeeded in immunizing animals against various antigens, including toxins, without producing an increase in the globulin fraction of the serum-proteins. In many infections and pathological conditions the globulin is increased, although frequently the total protein is diminished. During immunization, particularly against toxic antigens, waxy disease and hepatic haemorrhages occur, and it appears likely that pathological changes or disturbed metabolism may account in great measure for the alteration in the composition of the serum-protein observed during immunization. More recent work on the purification of antibodies has shown that specific activity is associated with a very small part of the immune serum and very little of the globulin increase which occurs on immunization can be ascribed to the accumulation of antibodies.

#### THE PURIFICATION AND CONCENTRATION OF ANTISERA.

An important practical application of the results obtained from the study of the distribution of antibodies in the serum of immunized animals has been the introduction of methods for the purification and concentration of therapeutic, especially antitoxic, sera.

The successful methods now so extensively used are based upon the pioneer work of Gibson (1906) and Banzhaf (1908, 1909, 1913, 1917 ; Banzhaf and Gibson, 1907). Since Gibson's original publication various modifications have been introduced with the varied objects of improving the yield, reducing the protein content of the final product, facilitating particular stages in the technical process such as filtering, and eliminating the tendency for opalescence and precipitation in the final product (Homer, 1916<sup>1, 2 & 3</sup>, 1917<sup>1, 2 & 3</sup>, 1918<sup>1 & 2</sup>, 1919<sup>1 & 2</sup> ; Banzhaf and Gibson, 1907 ; Banzhaf, 1908, 1909, 1913, 1917 ; Heinemann, 1916 ; Chick, 1914-15). The methods adopted vary in certain details, and it is only possible to give a general outline of the process. The whole question of the economical production of highly purified therapeutic sera is of such importance, both to manufacturers and clinicians, that concentration processes are continually under critical examination and improvements in technique are constantly being made.

The first important modification of Gibson's original process, which was concerned in the main with the separation of the pseudo-globulin fraction of antitoxic sera, was based on the experiments of Banzhaf (1908) who showed that such sera could be heated to 56 to 58° C. for several hours without loss of specific activity, while at the same time some of the soluble pseudo-globulin acquired the solubility properties of euglobulin and could thus be removed with the inactive euglobulin. It was also found that the presence of 0·5 per cent. phenol, or smaller quantities of tricresol, facilitated filtration, and that the presence of salts favoured the conversion of pseudo-globulin into its less soluble modification. Accordingly, in the first stage of the purification process it is customary to heat the slightly diluted serum or plasma, usually containing phenolic antiseptic and small concentrations of common salt or higher concentrations of ammonium sulphate, to 56° C. or slightly higher temperatures. The duration of the heating is determined by the temperature and the amount of salt and antiseptic present: if the preliminary heating has been conducted in the presence of 30 per cent. ammonium sulphate it is merely necessary to filter the heated mixture; otherwise 30 per cent. ammonium sulphate is added and the precipitate, consisting of euglobulin, denatured pseudo-globulin and a little antitoxin, is filtered off. The antitoxin contained in this fraction, usually small in amount, may be recovered. To the filtrate containing antitoxin, pseudo-globulin and albumin, ammonium sulphate solution is added to make the concentration of the salt 45 per cent., and the precipitate, consisting of pseudo-globulin and most of the antitoxin, is filtered off. The precipitate is pressed between cloths, dialysed until free from salts and 1 per cent. sodium chloride and antiseptic added. The finished product is stored for several weeks and finally filtered through germ-proof filters and distributed into containers.

In recent years the removal of the troublesome euglobulin has been attempted by other means. The preliminary heating is frequently omitted and the serum or plasma is made more dilute than formerly before fractionation. In many laboratories the product obtained, either by the original or modified methods, is redissolved and the purification process repeated. Preparations of concentrated antitoxins are now commonly available which, in regard to appearance, low protein content and high potency, are far superior to those current only a few years ago.

Other therapeutic sera are now concentrated by Banzhaf's process, the method as used for antitoxins being modified where necessary in accordance with the distribution of the antibody in the native antiserum, the salt precipitation limits, and the behaviour of the antibody when heated under different conditions. Scarlet fever antitoxin is purified by the same method as is used for diphtheria and tetanus antitoxins (Banzhaf, 1920-26). Homer (1920) states that the antibodies present in anti-dysentery and antimeningococcic sera are associated with the euglobulin-pseudo-globulin zone of the serum-proteins of immunized horses. Avery

(1915) found that dialysis, dilution and acidification, saturation with common salt or one-third saturation with ammonium sulphate removed some, but not all, of the protective antibodies occurring in antipneumococcus serum (Type I), and that complete precipitation was effected by 38 to 42 per cent. saturation with ammonium sulphate. Thus, the distribution of this antibody is different from that of the antitoxins. Banzhaf (1924-5) repeated and confirmed these observations and by a modification of his process for antitoxin concentration has evolved a practical method for the purification and concentration of antipneumococcus serum.

Sodium sulphate has been used for the purification of therapeutic sera by Brunner and Pinkus (1907), Sordelli (1923) and MacConkey (1924<sup>2</sup>).

Freund and Sternberg's (1899) observation that aluminium salts precipitate albumin and leave antitoxin in solution with the globulin was made use of by Seng (1899), but this method of effecting a primary separation of albumin has not been extensively employed.

Felton (1925<sup>1</sup>) showed that antipneumococcus serum simply diluted with 10 to 20 volumes of water yielded a larger amount of precipitate than normal horse-serum similarly treated, and that by this simple procedure some 90 per cent. of the inactive serum-proteins were removed and about 80 per cent. of the protective antibodies recovered in the water-insoluble fraction. In his later work (1925<sup>2</sup>, 1928<sup>1 & 2</sup>), the properties of the protective substance were investigated and methods for further purification sought for. Felton found that the protective substance is always associated with a water-insoluble protein, and although the substance resembles euglobulin it differs from the euglobulin of normal horse-serum in two important particulars : it has been obtained practically free from phosphorus and its isoelectric point is different from that of normal euglobulin. The protective substance, therefore, appears to be different from any protein hitherto described as occurring in normal horse-serum. Felton found that the protective substance was precipitated by 28 to 44 per cent. of ammonium sulphate [see Avery (1915) and Banzhaf (1924-5)]. In addition to the water-dilution method, Felton (1928<sup>3</sup>) has described a method of purification by means of sodium sulphate ; the final product is of high potency, stable, clear and almost colourless and is practically free from the substances causing febrile reactions which occur in imperfectly purified preparations.

In recent years the method of electrodialysis has been applied to the investigation of antitoxic and other antisera. In this method the serum is contained in the middle compartment of a three-chambered cell, the partition being effected by means of two semi-permeable membranes. The two end compartments carry platinum electrodes immersed in running distilled water. When a potential difference is established between the electrodes the ions of the serum pass to the electrodes, the equilibrium between the electrolytes and proteins is disturbed, and the globulins are precipitated. Euglobulin first separates, and Pauli (1924) made the

interesting observation that on prolonged electrodialysis pseudo-globulin is precipitated as well, and a globulin-free solution of albumin may be obtained. This method has been used by Ruppel (1923), Wernicke (1922, 1925) and Adolf (1924) for the purification and concentration of diphtheria antitoxin, and by Locke and Hirsch (1924) for haemolytic amboceptor. Adolf found that the precipitates produced in the early stages contained relatively little antitoxin ; as the conductivity decreased there was a sharp increase in the antitoxin content of the precipitate, and all the antitoxin was removed when precipitation was complete. Wernicke, however, reports that he was unable to remove all the antitoxin, even when the conductivity was decreased to values lower than those obtained by Adolf. The results of the examination of antitoxin by this method have shown, as salting-out methods have done, that diphtheria antitoxin is associated with the more soluble fraction of the pseudo-globulin.

#### ATTEMPTS AT THE ISOLATION OF ANTIBODIES.

It is generally agreed, and by no one more readily than those who have practical experience of salting-out methods, that while these are of the greatest service in effecting partial purification and concentration of antisera, their limitations are such as to render them unsuitable for the isolation of antibodies. A study of the most carefully conducted salt precipitation experiments provides little ground for hope that by such methods alone can antibodies be entirely freed from other constituents of serum.

Of the other methods available, those based upon the characteristic property of all antibodies of reacting with a specific antigen have been most extensively studied. It is well known that under suitable conditions antibodies can be removed from their solutions by absorption with specific antigen. The antigen-antibody combination can be washed and thus freed from a very large part of the serum-proteins. In the attempt to isolate antibodies from such starting material it is first necessary to dissociate the antigen and antibody and then separate the latter from the other constituents of the mixture.

The conditions governing the dissociation of antigen and antibody have been investigated by many workers. It has been shown that some dissociation of the combination of toxins with their specific antitoxins occurs on simple dilution, and Todd (1928) has also found that fowl-plague virus may be dissociated from its antiserum by the same means. Muir (1903) showed that haemolysin could be partially dissociated from its combination with red cells at 37° C., and that the process takes place more slowly at room temperature, while at 0° C. there is practically no dissociation. Landsteiner and Jagić (1903), Bail and Tsuda (1909) and Spät (1910) showed that antibody was split off from its combination at 42 to 45° C., and Hahn and Trommsdorf (1900) and Liebermann and Fenyvessy (1908) that antigen-antibody combinations could be dissociated by weak acids. Weinstein (1918) extracted the precipitate

produced by adding an extract of typhoid bacilli to antityphoid serum with weak alkali at 42° C. and demonstrated antibodies in solution.

Kosakai (1918) showed that haemolysin could be removed from sensitized red cells by means of isotonic cane-sugar solutions ; he considers that the reaction is completely reversed. By treatment of the haemolysin solution with ether Kosakai obtained active solutions of antibody containing only small amounts of protein. The methods of Kosakai were applied by Furuhata (1921) to the study of haemagglutinins and by Uchida (1927) to the study of haemolysin and the agglutinins and complement-fixing antibodies of antityphoid serum. Uchida's purified preparation of haemolysin contained traces of nitrogen, but failed to sensitize guinea-pigs anaphylactically : the activity remained unimpaired after digestion with trypsin for eighteen hours.

Huntoon and Etris (1921) investigated the recovery of agglutinins, bactericidal antibodies and the protective antibody of antipneumococcus serum from their combinations with antigen. They showed that reversal of the reaction was never complete and suggest that dissociation is governed by the dilution of the salts present, the nature of the salt varying with different antibodies. The protective antibody attached to pneumococcus antigen could be recovered by saccharose and dextrose solutions and by other means. Huntoon and Etris were able to prepare solutions of the pneumococcus antibody of high potency and containing so little protein that 5 c.cm. quantities sensitized guinea-pigs irregularly to subsequent injections of horse-serum. Huntoon, Masucci and Hannum (1921) carried out a chemical examination of these highly purified antibody solutions. A preparation containing 0.035 mgm. of nitrogen per c.cm. and free from phosphorus and sulphur gave none of the usual reactions for protein ; the antibody did not dialyse and showed no loss of activity, even after being subjected to the action of trypsin for a week at 37° C. Some of the material in the extract could be removed either by dialysis or dilution and acidification and the remaining solution was found to have lost little in potency. The solubility properties of the antibody indicated that it was unlike both euglobulin and pseudo-globulin ; it resisted the action of weak alkalis and weak acids, was not injured by heating to 60° C., but was destroyed by heating at higher temperatures.

Ottenberg (1923) and Ottenberg and Stenbuck (1923, 1924, 1925) effected a further purification of antibody solution by means of minute quantities of copper chloride at definite hydrogen-ion concentrations. Solutions of typhoid agglutinin were obtained containing only 0.0042 mgm. of nitrogen per c.cm. The method is applicable to the further purification of pneumococcus antibody solutions prepared by Huntoon's method.

Locke and Hirsch (1925) found that the stromata obtained after lysis of sheep's erythrocytes with perfectly fresh rabbit antisheep-serum contained from 90 to 99 per cent. of the haemolysin present in the original antiserum. After repeated washing the haemolysin-stromata precipitate was extracted with ether, washed, and finally extracted with N/1000

sulphuric acid; on neutralization of the acid extract a white insoluble precipitate was obtained which yielded solutions of haemolysin which in some cases contained little more than a twenty-thousandth of a milligram of nitrogen per unit of haemolysin, representing a purification of over 100,000 per cent.

Preparations of diphtheria antitoxin, far more highly purified than any which have been obtained by salting-out methods, have recently been obtained from the specific precipitates produced in neutral, or nearly neutral, mixtures of diphtheria toxin and antitoxin. Ramon (1923) recovered from these precipitates 75 per cent. of the antitoxin units employed and effected the removal of over 99 per cent. of the impurities. Locke and Main (1926), by the isoelectric fractionation of washed toxin-antitoxin floccules which had been extracted with ether, obtained preparations of antitoxin containing only 0.0005 mgm. of nitrogen per unit of antitoxin; their purest preparations, however, sensitized guinea-pigs to normal horse-serum. The writer also found that the toxin-antitoxin precipitates investigated by him (1926), even after repeated washing with salt solution, sensitized guinea-pigs to normal horse-serum.

While the question of their chemical nature remains undecided, recent work on purification and attempted isolation has advanced our knowledge of antibodies in many ways. Solutions of antibodies containing far smaller amounts of impurities than hitherto have been prepared and the effect of chemical and physical agencies on their biological activity has been investigated. These studies have shown that antibodies are complex substances of high molecular weight having the general properties of colloids. They withstand short exposure to low concentrations of acids and alkalis, resist heating to 60° C., but are destroyed at higher temperatures. They contain nitrogen, but appear to be devoid of sulphur and phosphorus. The difficulty of removing all serum-protein, and the lack of criteria of purity for antibodies, retards the solution of the problem as to whether antibodies are proteins. The best evidence yet available in support of the non-protein nature of antibodies has been furnished by Huntoon. Some of his solutions contained nitrogen which, if reckoned as protein, was present in concentrations sufficiently large to give many of the reactions of protein, and they failed to do so. Highly concentrated solutions of antibodies sensitized guinea-pigs irregularly to normal and immune horse-serum and the antibody resisted the action of trypsin. It is possible that failure to sensitize guinea-pigs and resistance to trypsin may be due to racemization, but it is significant that the specific properties of the antibody remained after long exposure to alkali and trypsin.

It has long been recognized that the activity of an antiserum is associated with a small part of the total solid present, and recent attempts to isolate antibodies has emphasized how very small these quantities are: they have shown that antibodies must be classed among the most biologically active substances known. Herein lies one of the main difficulties in the way of further progress in this field. It is obvious that in order to

secure adequate amounts of material for chemical examination very large quantities of antiserum will be required; by the application of the methods recently introduced a very large amount of inert protein can be separated, but more perfect purification awaits the discovery of new methods. One fact of interest has emerged from the intensive study of antipneumococcus serum; as a result of immunization a new substance, different in its properties from normally occurring constituents, appears in the serum. Whether this substance is non-protein in nature or a modified globulin is not known.

## REFERENCES.

ADOLF, M., 1924, *Klin. Wschr.*, **3**, 1214.  
 ANDERSON, J. F., 1910, *Bull. U.S. Hyg. Lab.*, No. 66, 1.  
 AVERY, O. T., 1915, *J. Exp. Med.*, **21**, 133.  
 BAIL, O. & TSUDA, K., 1909, *Z. ImmunForsch.*, Tl. I, Orig., **1**, 546.  
 BAIVY, A., 1926, *C.R. Soc. Biol.*, Paris, **95**, 737.  
 BAKER, A. H., 1925, *Brit. J. Exp. Path.*, **6**, 201.  
 BANZHAF, E. J., 1908, *Proc. Soc. Exp. Biol.*, N.Y., **5**, 8; 1909, *Coll. Stud. Dept. Hlth., N.Y. Cy.*, **4**, 230; 1913, *ibid.*, **7**, 114; 1911, *Johns Hopkins Hosp. Bull.*, **22**, 106; 1917, *Proc. N.Y. Path. Soc.*, **17**, 31; 1920-26, *Coll. Stud. Dept. Hlth., N.Y. Cy.*, **10**, 367; 1924-5, *Proc. Soc. Exp. Biol.*, N.Y., **22**, 329.  
 BANZHAF, E. J. & FAMULENER, W., 1914-15, *Coll. Stud. Dept. Hlth., N.Y. Cy.*, **8**, 208.  
 BANZHAF, E. J. & GIBSON, R. B., 1907, *J. Biol. Chem.*, **3**, 253.  
 BANZHAF, E. J., SUGIURA, K. & FALK, K. G., 1917, *J. Immunol.*, **2**, 125.  
 BARONI, V. & JONESCU-MIHAIESTI, C., 1910<sup>1</sup>, *C.R. Soc. Biol.*, Paris, **68**, 393; 1910<sup>2</sup>, *ibid.*, **69**, 273.  
 BELJAEFF, W., 1903, *Zbl. Bakt.*, Abt. I, Orig., **33**, 369.  
 BERG, W. N., 1921, *J. Amer. Med. Ass.*, **76**, 1820.  
 BERGER, W., 1922, *Schweiz. med. Wschr.*, **52**, 225; 1922, *Z. ges. exp. Med.*, **28**, 1.  
 BITTER, L., 1922, *Zbl. Bakt.*, Abt. I, Orig., **87**, 560.  
 BRUNNER, J. & PINKUS, S. N., 1907, *Biochem. Z.*, **5**, 381.  
 CHICK, H., 1914-15, *J. Path. Bact.*, **19**, 131.  
 DARÁNYI, J., 1928, *J. Immunol.*, **15**, 521.  
 DOERR, R. & BERGER, W., 1921, *Z. Hyg. InfektKr.*, **93**, 147.  
 DOERR, R. & HALLAUER, C., 1926, *Z. ImmunForsch.*, **47**, 363.  
 FELTON, L. D., 1925<sup>1</sup>, *J. Infect. Dis.*, **37**, 199; 1925<sup>2</sup>, *ibid.*, **37**, 309; 1928<sup>1</sup>, *ibid.*, **42**, 248; 1928<sup>2</sup>, *ibid.*, **42**, 256; 1928<sup>3</sup>, *ibid.*, **43**, 543.  
 FELTON, L. D. & BAILEY, G. H., 1926, *J. Immunol.*, **11**, 197.  
 FREUND, E. & STERNBERG, C., 1899, *Z. Hyg. InfektKr.*, **31**, 429.  
 FURUHATA, T., 1921, *Japan Med. Wld.*, **1**, No. 6, p. 1.  
 GIBSON, R. B., 1906, *J. Biol. Chem.*, **1**, 161.  
 GIBSON, R. B. & BANZHAF, E. J., 1910, *J. Exp. Med.*, **12**, 411.  
 GIBSON, R. B. & COLLINS, K. R., 1907, *J. Biol. Chem.*, **3**, 233.  
 GLAESNER, K., 1905, *Z. exp. Path. Ther.*, **2**, 154.  
 GLENNY, A. T., 1913, *J. Hyg., Camb.*, **13**, 63.  
 GLENNY, A. T., POPE, C. G., WADDINGTON, H. & WALLACE, U., 1925<sup>1</sup>, *J. Path. Bact.*, **29**, 33; 1925<sup>2</sup>, *ibid.*, **29**, 36.  
 HAHN, M. & TROMMSDORF, R., 1900, *Münch. med. Wschr.*, **47**, 413.  
 HARDY, W. B. & GARDINER, E., 1910, *Proc. Physiol. Soc., J. Physiol.*, **40**, lxviii.  
 HARTLEY, P., 1914<sup>1</sup>, *Mem. Dept. Agric. India*, **4**, 171, 205, 220; 1914<sup>2</sup>, *Biochem. J.*, **8**, 541; 1925, *Brit. J. Exp. Path.*, **6**, 112, 180; 1926, *ibid.*, **7**, 55.  
 HARTLEY, P., EAGLETON, A. J. & OKELL, C. C., 1923, *J. Path. Bact.*, **26**, 53.  
 HEINEMANN, P. G., 1916, *J. Infect. Dis.*, **19**, 433.  
 HEUER, G., 1922, *Zbl. Bakt.*, Abt. I, Orig., **88**, 380.  
 HOMER, A., 1916<sup>1</sup>, *Biochem. J.*, **10**, 280; 1916<sup>2</sup>, *J. Hyg., Camb.*, **15**, 388; 1916<sup>3</sup>, *ibid.*, **15**, 580; 1917<sup>1</sup>, *Biochem. J.*, **11**, 21; 1917<sup>2</sup>, *ibid.*, **11**, 277; 1917<sup>3</sup>, *ibid.*, **11**, 292; 1918<sup>1</sup>, *ibid.*, **12**, 190; 1918<sup>2</sup>, *J. Hyg., Camb.*, **17**, 51; 1919<sup>1</sup>, *Biochem. J.*, **13**, 45; 1919<sup>2</sup>, *ibid.*, **13**, 56; 1920, *ibid.*, **14**, 42.

## 266 EFFECT OF PHYSICAL AND CHEMICAL AGENCIES ON THE PROPERTIES OF ANTIGENS AND ANTIBODIES

HURWITZ, S. H. & MEYER, K. F., 1916, *J. Exp. Med.*, **24**, 515.  
HURWITZ, S. H. & WHIPPLE, G. H., 1917, *J. Exp. Med.*, **25**, 231.  
HUNTOON, F. M., 1921, *J. Immunol.*, **6**, 117.  
HUNTOON, F. M. & ETRIS, S., 1921, *J. Immunol.*, **6**, 123.  
HUNTOON, F. M., MASUCCI, F. & HANNUM, E., 1921, *J. Immunol.*, **6**, 185.  
JOACHIM, J., 1903, *Pfluegers Arch.*, **93**, 558.  
JONES, F. S., 1927, *J. Exp. Med.*, **46**, 291.  
JOOS, A., 1903, *Zbl. Bakt.*, Abt. I, Orig., **33**, 762.  
KAPSENBERG, G. & RISPENS, T. E., 1927, *Z. ImmunForsch.*, **52**, 227.  
KIRKBRIDE, M. B. & MURDICK, P. P., 1927, *J. Immunol.*, **14**, 235.  
KOSAKAI, M., 1918, *J. Immunol.*, **3**, 109.  
KRAUS, R. & JOACHIM, J., 1904<sup>1</sup>, *Zbl. Bakt.*, Abt. I, Orig., **36**, 662; 1904<sup>2</sup>, *ibid.*, **37**, 73  
KRAUS, R. & V. PIRQUET, C., 1902, *Zbl. Bakt.*, Abt. I, Orig., **32**, 60.  
KRUMWIEDE, C. & BANZHAF, E. J., 1921, *J. Infect. Dis.*, **28**, 367.  
LANDSTEINER, K. & JAGIČ, N., 1903, *Münch. med. Wschr.*, **50**, 764.  
LANGSTEIN, L. & MAYER, M., 1904, *Beitr. chem. Physiol. Path.*, **5**, 69.  
LEDINGHAM, J. C. G., 1907, *J. Hyg., Camb.*, **7**, 65.  
LIEBERMANN, L. V. & FENYVESSY, B. V., 1908, *Zbl. Bakt.*, Abt. I, Orig., **47**, 274.  
LOCKE, A. & HIRSCH, E. F., 1924, *J. Infect. Dis.*, **35**, 519; 1925, *ibid.*, **37**, 449.  
LOCKE, A. & MAIN, E. R., 1926, *J. Infect. Dis.*, **39**, 484.  
LOCKE, A., MAIN, E. R. & HIRSCH, E. F., 1926, *J. Infect. Dis.*, **39**, 126.  
MACCONKEY, A. T., 1912, *J. Hyg., Camb.*, **12**, 511; 1924<sup>1</sup>, *ibid.*, **22**, 473; 1924<sup>2</sup>, *ibid.*, **22**, 413.  
MADSEN, T. & STRENG, O., 1909, *Z. physik. Chem.*, **70**, 263.  
MADSEN, T. & WALBUM, L. E., 1917, *Commun. Inst. sérthérap.*, Copenh., **9**, 1.  
MARTIN, C. J., 1896, *J. Path. Bact.*, **3**, 507.  
MELLANBY, J., 1907, *J. Physiol.*, **36**, 288; 1908, *Proc. Roy. Soc., B.*, **80**, 399.  
MEYER, K. F., HURWITZ, S. H. & TAUSSIG, L., 1918, *J. Infect. Dis.*, **22**, 1.  
MODERN, F. & WERNICKE, R., 1929, *C.R. Soc. Biol.*, Paris, **102**, 452.  
MOLL, L., 1903, *Beitr. chem. Physiol. Path.*, **4**, 578; 1906, *Z. exp. Path. Ther.*, **3**, 325.  
MUIR, R., 1903, *Lancet*, Lond., ii, 100, 446.  
MÜLLER, F., 1898, *Zbl. Bakt.*, Abt. I, **24**, 251, 316.  
MÜLLER, P. T., 1905, *Beitr. chem. Physiol. Path.*, **6**, 454.  
NOÜY, P. L. DU, 1923, *J. Exp. Med.*, **37**, 659.  
OLITZKI, L., 1928, *Zbl. Bakt.*, Abt. I, Orig., **106**, 267; 1928, *Z. ImmunForsch.*, **58**, 244.  
ORNSTEIN, O., 1928, *Klin. Wschr.*, **7**, 1081.  
OTTERBERG, R., 1923, *Proc. Soc. Exp. Biol.*, N.Y., 1923, **21**, 14.  
OTTERBERG, R. & STENBUCK, F., 1923-4, *Proc. Soc. Exp. Biol.*, N.Y., 1923, **21**, 303; 1924-5, *ibid.*, **22**, 211, 215.  
PAULI, W., 1924, *Klin. Wschr.*, **3**, 1; 1924, *Biochem. Z.*, **152**, 355.  
PICK, E. P., 1902, *Beitr. chem. Physiol. Path.*, **1**, 351, 393, 445.  
PRÖSCHER, E., 1902, *Münch. med. Wschr.*, **49**, 1176.  
RAMON, G., 1923, *C.R. Soc. Biol.*, Paris, **88**, 167; 1923, *C.R. Acad. Sci.*, Paris, **178**, 267.  
REITSTÖTTER, J., 1920, *Z. ImmunForsch.*, Tl. I, Orig., **30**, 507.  
REYMANN, G. C., 1924, *Z. ImmunForsch.*, **39**, 15.  
RUPPEL, W. G., 1923, *Deuts. med. Wschr.*, **49**, 40.  
RUPPEL, W. G., ORNSTEIN, O., CARL, J. & LASCH, G., 1922, *Z. Hyg. InfektKr.*, **97**, 188.  
SCHMIDT, E. S. & SCHMIDT, C. L. A., 1917, *J. Immunol.*, **2**, 343.  
SCOTT, W. M., 1911, *J. Path. Bact.*, **16**, 148.  
SENG, W., 1899, *Z. Hyg. InfektKr.*, **31**, 513.  
SHACKELL, L. F., 1909, *Amer. J. Physiol.*, **24**, 325.  
SORDELLI, A., 1923, *C.R. Soc. Biol.*, Paris, **89**, 451.  
SPÄT, W., 1910, *Z. ImmunForsch.*, Tl. I, Orig., **7**, 712.  
STRENG, O., 1909, *Z. Hyg. InfektKr.*, **32**, 281.  
TADOKORO, T. & NAKAYAMA, Y., 1920, *J. Infect. Dis.*, **26**, 8.  
TODD, C., 1928, *Brit. J. Exp. Path.*, **9**, 244.  
UCHIDA, S., 1927, *J. Infect. Dis.*, **40**, 588, 597.  
WEINSTEIN, I., 1918, *J. Immunol.*, **3**, 17.  
WERNICKE, R., 1922, *C.R. Soc. Biol.*, Paris, **87**, 1041; 1925, *ibid.*, **93**, 879.

## CHAPTER VIII. BACTERICIDAL ACTION.

BY R. MUIR (UNIVERSITY OF GLASGOW);  
WITH A SECTION BY C. H. BROWNING (UNIVERSITY OF GLASGOW).

### Antibacterial Sera.

IT is to the work of Pfeiffer from 1894 onwards that we owe our earliest knowledge of specific antibodies developed against bacteria. Before that time it had been established by various observers that there might be produced by an antiserum a passive immunity which was not due to antitoxins. For example, Behring and Nissen (1890) found that the serum of a guinea-pig immunized against *V. metchnikovi* possessed increased bactericidal action on that organism, and Metchnikoff (1893) showed that the preventive effect of an anticholera serum was not due to its containing antitoxin. Fraenkel and Sobernheim (1894) found that such a serum had increased bactericidal action on the cholera vibrio, and that passive immunity could be conferred by it even after it had been heated at 70° C. for an hour. The fundamental facts established by Pfeiffer and his co-workers were that when cholera vibrios are introduced into the peritoneum of an animal immunized against the vibrio, the organisms undergo an extracellular lysis, and that when passive immunity is conferred by the serum of an animal actively immunized a similar lysis occurs in the peritoneal cavity of the passively immunized animal. This extracellular lysis became generally known as *Pfeiffer's phenomenon* or *bacteriolysis*. Pfeiffer regarded it as due to a *specific antibody* in the serum of the immunized animal and thus the reaction could be used for diagnosis, the lytic effect being exerted only towards the organism used in immunization. He believed that the antibody, which is not destroyed at 55° C., existed in the serum in an inactive state and that it was activated by the cells of the animal, especially by the endothelial cells of the peritoneum. Metchnikoff (1895) showed, however, that when the test was carried out *in vitro* activation could be effected by the addition of fresh peritoneal fluid, and a little later the same result was obtained by Bordet (1895) by the addition of normal serum. The latter added many facts of importance, and ultimately it came to be recognized that in this type of immunity the essential feature is the development of an antibody which does not act by itself, but which with the aid of alexin or complement leads to the death and solution of the organisms. Such an antibody is usually spoken of as an *immune-body, sensitizer (substance sensibilisatrice), or amoceptor*. (It is to be noted that Bordet uses the term alexin in the same sense as complement, and that the term does not exactly correspond

with Buchner's alexin, the latter indicating the bactericidal substance of normal serum which is now known in many instances to consist of normal immune-body and complement.)

Apart from the effect of an immune-serum in producing bacteriolysis or in leading to protection, it is now recognized that as a general rule the injection of a bacterium in suitable amounts into an animal stimulates the production of antibodies which may act in a corresponding way to those concerned in bacteriolysis but without producing this effect ; that is, in combination with the homologous bacteria they lead to the fixation of complement. Thus, we speak of *complement-fixing* immune-bodies when such an effect without bactericidal action is produced (Chapter X). This, as a rule, means simply that the particular bacterium is not sensitive to the action of complement, the action of the immune-body being essentially to bring complement into union with the bacterium. A similar condition sometimes holds in haemolysis (Chapter IX) when a complement does not lyse sensitized corpuscles. When an antiserum does produce bactericidal action it remains to be determined whether all the complement fixed is concerned in the bactericidal process, and whether or not the bactericidal immune-body is identical with the complement-fixing immune-body. This question will be discussed later.

Bacteriolysis or bactericidal action is, however, seen in the case of a relatively small proportion of organisms as will be detailed below, and a great many species, such as the pyogenic cocci and many other Gram-positive organisms, are not susceptible to the bactericidal action of serum, whether it be a normal serum or that of an animal immunized against the particular organism. In many such instances, however, the immune-serum, e.g. an antistreptococcic or antipneumococcic serum, contains antibodies which lead to the phagocytosis of the corresponding bacteria. In this case the protection conferred is due not to extracellular lysis but to destruction of the bacteria within the leucocytes, the ingestion of the organisms being effected by the specific antibodies. The antibodies in question are known as *bacteriotropins* or *opsonic immune-bodies* and such antisera are said to act by their increased *opsonic* effect, the mechanism of which will be considered in Chapter XI. When an antiserum is produced by injection of bacteria, the antibodies which it contains may be demonstrated by their effects in producing clumping or agglutination of the corresponding bacteria or by producing a precipitate with a filtrate of culture. Although these two antibody reactions are not concerned in the destruction of the bacteria, they are of great service in the study of immunity reactions; they also are considered later (Chapters XII and XIII).

Lastly, in certain instances the preventive property of an antiserum has not been shown to be due to the presence of antibodies for the bacteria employed as antigens, and consequently its specific action is not removed by absorption with a suspension of the corresponding bacteria. The outstanding example of this type is an anti-anthrax serum, to which further reference is made below.

If we consider as a whole the sera developed in response to the injection of bacteria, as contrasted with antitoxic sera, we see that the antibodies which they contain may be demonstrated either by their giving rise to passive immunity, or by certain reactions *in vitro*, or again in certain instances in both of these ways. So far as their mode of action in leading to the death of the corresponding bacteria is concerned, they may be arranged according to the following scheme :

(1) Antisera with specific antibodies, the latter being absorbable by the corresponding bacteria : (a) antibody along with complement leading to death of the corresponding bacteria with or without lysis—bactericidal action ; (b) antibody leading to phagocytosis of bacteria, with or without the aid of complement—opsonic or bacteriotropic action (Chapter XI).

(2) Antisera which lead to protection without the presence of antibody absorbable by the corresponding bacteria and without the co-operation of ordinary complement.

Evidence regarding the substances concerned in bactericidal action was extended by the discovery by Bordet (1898) that a haemolytic serum could be developed by the injection of the corpuscles of one animal into another of different species and that in this case also immune-body and complement were concerned in the lysis. Study of haemolytic sera showed that other facts established with regard to bacteriolysis held in the case of haemolysis, and brought out additional information with regard to the relations of immune-body and complement. The advantage of the haemolytic method is that observations can be made more easily and with greater precision. The general result has been to show that although some differences exist there is a close correspondence between the lytic processes in the two cases. We shall give first of all an account of the bactericidal action of antisera and thereafter the properties of haemolytic sera will be considered. The properties of complement, which is concerned in both cases, will then be dealt with in greater detail.

### Bactericidal Action.

The essential feature of an antiserum with bactericidal action is the presence in it of specific antibody (immune-body) which leads to the union of complement. Such a serum may lead to actual destruction or solution of the bacteria, bacteriolysis, or merely to death of the bacteria ; and it is to be noted that certain sera may possess a bactericidal action without bacteriolytic action. Further, although the killing of the bacteria is the effect usually tested for, all degrees of inhibition of growth up to actual death may be effected by the antiserum. The serum has also a preventive action as shown by the protection conferred on an animal by injection of the serum. All these properties when present can be estimated by quantitative methods.

It is to be noted that in the development of an antiserum there is a progressive formation of immune-body without corresponding increase in

complement, and accordingly the potency of the serum cannot be estimated by merely using the fresh serum in varying dilutions ; a proper adjustment between immune-body and complement is necessary for the optimum bactericidal effect. Further, as will be shown below, excess of immune-body may have an inhibitory effect and an antiserum used in the fresh state may actually be without bactericidal action (p. 275).

#### *Pfeiffer's Phenomenon—Bacteriolysis.*

This phenomenon was first described by Pfeiffer and Issaeff (1894<sup>1</sup> & 2) in the case of the cholera vibrio, and was thereafter the subject of a series of papers by Pfeiffer and his co-workers. When first discovered it was used by them as a diagnostic agent between allied organisms, but its use in this way has been supplanted by other serum reactions since discovered, e.g. agglutination and complement fixation, as these latter are so much more readily applied. All these reactions have a similar diagnostic significance, but in the period following Pfeiffer's discovery bacteriolysis was largely used in distinguishing the cholera vibrios from allied forms. A little later it was applied to the typhoid bacillus by Pfeiffer (1895<sup>2</sup>), Pfeiffer and Kolle (1896<sup>2</sup>) and by Löffler and Abel (1896), who also used *B. coli*. Details of the method are given in another place, but some points with regard to its use may be referred to.

In the first place the organism to be tested must not be in an avirulent state ; it must be able to multiply in the peritoneum of the guinea-pig and produce a fatal result. Some avirulent strains of the cholera and typhoid organisms undergo lysis even in a normal serum or in the peritoneal cavity without antiserum. And there is general agreement amongst observers that the more virulent the strain used the more clearly is the specificity of the serum brought out. The next point is that in any case there is a certain amount of antiserum which gives the optimum result. This is especially important when the test is carried out *in vitro*. It was shown by Neisser and Wechsberg (1901) that excess of immune-body may interfere with the bactericidal action of complement and with bacteriolysis. This action does not hold in the same way in *in-vivo* experiments, as complement is supplied in varying amounts, but failure to produce protection by excess of antibody is also met with, as was shown by Pfeiffer (1895<sup>1</sup>) and by Löffler and Abel (1896). The same phenomenon was observed by Leclainche and Morrel (1901) in the case of an antiserum to the *Vibron septique*. Such a result may be due to inhibitory action of antibody in excess as in *in-vitro* experiments (p. 274), but it has also been ascribed to a rapid setting free of endotoxins by the lysis and thus to rapid poisoning of the animal.

The guinea-pig is the test animal ordinarily used for Pfeiffer's phenomenon, an animal of 200 to 250 gm. being employed ; and guinea-pig's serum is especially suitable for the *in-vitro* test owing to its richness in complement and in view of the fact that it acts well in association with mammalian immune-bodies generally. With avian immune-bodies,

however, mammalian complement may fail to act. For example, Wechsberg (1902) found that an immune-body from the pigeon for *V. metchnikovi* was complemented by pigeon's serum with resulting lysis, but not by rabbit's serum, and vice versa. This is an example of a general law as to the relationships of avian and mammalian immune-bodies to complement, but there are exceptions to the rule (Chapter IX).

Pfeiffer's phenomenon occurs alike in an actively or passively immunized animal and also when the immune serum is injected along with the organisms into a normal animal, this last being the procedure in testing the potency of an antiserum. The stages in the process can be followed by withdrawing from time to time small quantities of fluid from the peritoneal cavity by capillary pipettes. In the case of the cholera vibrios it is found on microscopic examination that within a few minutes after the organisms have been introduced into the peritoneum the vibrios become altered in shape and appearance. They become swollen and rounded, this change being spoken of as spherulation, finely granular in appearance, and then undergo solution. Motility is lost at an early stage, but some of the spherulated forms may still show some movement. The changes can be readily followed in stained preparations made at different times. The rounded forms at first stain deeply with basic stains, but this is followed by a loss of affinity for the stain and ultimately the organisms become disintegrated and are not recognizable (for details of morphological changes see Radziewsky, 1901; Kraus, 1928). Lysis of the cholera organism may be complete within half an hour, but in the case of the typhoid bacillus the process takes considerably longer, from 1 to 2 hours. When the process is incomplete and organisms escape solution, these may be taken up by phagocytes and destroyed by their action. In cases where protection is imperfect progressive multiplication of the organisms may follow an initial partial destruction.

Pfeiffer adopted as the immunity unit or titre of an antibacterial serum (in conformity with Ehrlich's method) the minimum amount of serum which will produce bacteriolysis and protect a guinea-pig of 200 to 250 gm. weight against ten times the fatal dose. In cases where an anti-serum does not lead to bacteriolysis (e.g. antipneumococcic serum) its preventive properties can be accurately measured by similar methods (see Armstrong, 1925).

It is to be noted that comparatively few organisms are susceptible to direct bactericidal action and fewer still to bacteriolytic action. The cholera vibrio and its allies, e.g. *V. metchnikovi*, are especially susceptible to the lytic process, so also is the typhoid bacillus. The paratyphoid bacilli are less susceptible and the bacilli of the *coli* and dysentery groups are less so still, but considerable variations are met with amongst different strains. *B. pyocyaneus* also undergoes bacteriolysis under the action of an antiserum.

As already stated, all the work on the subject has gone to show that Pfeiffer's reaction is specific in the sense already explained, although the

minor effects on allied organisms are less easily demonstrated than in the case of other reactions. It is interesting to note in this connection that at an early date Löffler and Abel (1896), using Pfeiffer's reaction, showed that an antityphoid serum had some effect on *B. coli* and that the converse held good. The origin of bactericidal antibodies is discussed in Chapter VI (p. 213) (see Pfeiffer and Marx, 1898).

The in-vitro test is made by means of hanging-drop preparations which are afterwards incubated. Bordet used a suspension of a young culture in bouillon or 0·6 per cent. sodium chloride. To a drop of the suspension was added a drop of antiserum and a mixture was made; a drop of normal serum was then added to a drop of the mixture. But in any given case adjustment of amounts will be necessary for the best results. By this method he showed that results in all essential respects similar to those by Pfeiffer's method were obtained, and in particular that the reaction was specific. He established with precision the dual nature of the bactericidal action—the co-operation of the preventive substance (immune-body) and the alexin or complement of normal serum; in short, he established the fundamental facts now recognized. He noted also that the degree of lysis varied with different normal sera, some only changing the organisms to deeply staining granules, others causing complete dissolution.

While Pfeiffer's reaction and in-vitro bacteriolysis depend essentially on the same mechanism, viz. the action of complement along with a specific immune-body, a strict parallelism between the two phenomena may not be obtained. For example, lysis as a rule occurs more readily in the peritoneal cavity than when the test is made *in vitro*; in fact, in the case of the latter lysis is often incomplete. This is seen especially with the organisms less sensitive to the lytic process, e.g. *B. paratyphosus* (Bezzola, 1909). Again, Töpfer and Jaffé (1906) found that Pfeiffer's reaction was given in greater degree by the sera of typhoid convalescents and immunized animals than was bactericidal action *in vitro*; whereas during the course of typhoid fever the opposite was the rule. They concluded from their observations that Pfeiffer's reaction gave a more reliable indication of the true state of immunity.

The inhibiting action of normal serum on bacteriolysis was the subject of a series of investigations by Pfeiffer and Friedberger (1905, 1906). They removed the natural immune-body of a normal serum by absorbing it with a bacterium, the serum being afterwards centrifuged, and then found that the treated serum had an antagonistic effect when added along with the bacteria and the antiserum in the Pfeiffer reaction. They found that this effect was not due to the presence of free bacterial receptors in the treated normal serum nor was it due to anticomplement or anti-immune-body. The subject is one of considerable complexity and the true meaning of this phenomenon of inhibition has not been satisfactorily determined.

The work of Pfeiffer and others thus clearly demonstrated the extra-cellular destruction and solution of bacteria and was followed by much

controversy regarding the so-called humoral and cellular theories of immunity, the details of which have now a subordinate importance. As is well known, Metchnikoff upheld the importance of the phagocytes in the process by maintaining that the alexin concerned in bacteriolysis was set free by leucocytes on their disintegration. This view was apparently supported by the results of Gengou (1901), which went to show that plasma had little bactericidal power as compared with serum and that alexin did not exist preformed in the circulating blood. Gengou's view has, however, not been established ; in fact, we may say that it has been demonstrated by many subsequent researches that free complement is present in plasma and that the latter has full bactericidal powers (see papers by Lambotte, 1903 ; Watanabe, 1919 ; Morrison, 1922). Moreover, bacteria susceptible to lysis and also sensitized erythrocytes when introduced into the circulating blood undergo lysis in a manner similar to what is seen *in vitro*. Lippmann (1916) has shown that in guinea-pigs whose leucocytes had been largely destroyed by thorium-X, Pfeiffer's phenomenon occurred in the peritoneal cavity just as it did in the case of a normal animal. On the other hand, with an antipneumococcic serum whose preventive action depended upon bacteriotropins the protection obtained in a normal animal did not occur in the treated animal owing to the deficiency of leucocytes. There does not appear to be any satisfactory evidence that complement is formed by leucocytes (see Petrie, 1904), nor, in fact, has its origin been traced to any particular type of cell (Chapter X). This conclusion, of course, does not apply to other bactericidal substances or leukins formed by leucocytes (p. 284).

#### *Anti-Immune-Bodies.*

It was shown by Pfeiffer and Friedberger (1902<sup>1</sup>, 1903) that the injection of an anticholera serum from one animal into another of different species resulted in the formation of an anti-immune-body in the blood of the latter. They came to the conclusion that the anti-immune-body acts by combining with the cytophile group of the amboceptor and thus prevents its union with the bacteria. This conclusion presents certain theoretical difficulties and still more does the fact established by them, that a cholera anti-immune-body acts also against a typhoid immune-body. The subject was further investigated by Shibayama and Toyoda (1906), who arrived at other conclusions. They found that the anti-immune-body in no way interfered with the union of immune-body with the bacteria, but, on the other hand, that after union had taken place it prevented the union of complement. This result is in accordance with what has been established in the case of haemolysis. The existence of anti-immune-bodies in the strict sense has, however, now been called in question and the subject is discussed below (Chapter IX). The development of the antagonistic action, whatever its nature may be, on the injection of heterologous antiserum has a bearing on the short duration of passive immunity conferred by such a serum. The subject was investigated by

Pfeiffer and Friedberger (1904) and also by Schütze (1904), who injected bactericidal sera from different animals into one species and found that the immunity conferred by heterologous sera was of a shorter duration than that conferred by a homologous serum. In the latter case, of course, there is no development of 'anti-immune-body'. The immunity, however, was of considerably shorter duration than active immunity (see also Levin, 1909).

*Deviation of Complement : Neisser-Wechsberg Phenomenon.*

Neisser and Wechsberg (1901) showed that efficient bactericidal action of an immune-serum depends upon certain proportions between immune-body and complement, and especially that excess of immune-body interferes with bactericidal action. If in bactericidal experiments carried out by plating, the number of bacteria and the amount of complement be kept constant, and if tests be made with increasing amounts of immune-body, it is found that a minimal dose of immune-body is necessary to produce bactericidal effect, that when the immune-body is increased the bactericidal action becomes complete, and that on further increase the bactericidal action diminishes and disappears. There is thus an intermediate zone of efficiency of the action of immune-body ; in other words, with excess as well as with deficiency of immune-body, bactericidal action does not occur. They showed further that when a normal serum has bactericidal action on a particular bacterium, this can be annulled by the addition of a certain amount of homologous antiserum. The explanation of the phenomenon of inhibition given by Neisser and Wechsberg is based on Ehrlich's amboceptor hypothesis, according to which direct union of immune-body and complement takes place at warm temperatures. If the immune-body present is not more than can be taken up by the bacteria, all the molecules of combined immune-body will receive complement and thus bactericidal effect will follow. If, on the other hand, the immune-body is in excess so that a proportion remains uncombined, the complement will be distributed between the combined and the free immune-body and some of the former will not receive complement ; thus, bactericidal action will be interfered with. This will be the case provided (*a*) that the free immune-body has as great affinity for complement as the combined immune-body has, or (*b*) that the free immune-body has a greater affinity. This explanation, which rests on a supposed deviation of complement from the bacteria by excess of immune-body, became the subject of criticism, and other explanations were put forward.

Gay (1905) described a somewhat similar phenomenon in the case of haemolysis. He showed that if there is added to red corpuscles a small amount of serum from the same animal as that supplying the corpuscles, then an excess of immune-body may interfere with lysis by the minimum haemolytic dose of complement. The antiserum contains some serum antibody as well as haemolytic antibody, and the former combines with the

serum-antigen added and leads to fixation of complement, so that it is not available for lysis. Gay considered that in the case of bactericidal serum the precipitinogen set free from the bacteria interacts in a similar way with its antibody in the immune-serum and thus fixation of complement results. Agglutination may modify the results of bactericidal action, but the view that the Neisser-Wechsberg phenomenon is due to it was disproved by the observations of Lipstein (1902) and of Buxton (1904-5).

The inhibition of bacteriolysis by excess of immune-body may sometimes be seen in the case of a *fresh* immune-serum, such a serum being devoid of bactericidal action (Buxton, 1904-5; Thjøtta, 1920). This, however, is not always the case. Buxton found complete inhibition of bactericidal action in the case of a fresh antityphoid serum, whereas it was absent in an anticholera serum. Further, he showed that when a fresh antiserum is without bactericidal action the Neisser-Wechsberg phenomenon appears when it is diluted, there being then a pro-zone, a killing zone and a post-zone, according to the amounts of serum used. He made also the important observation that a fresh antityphoid serum without effect on typhoid bacilli is practically as bactericidal towards the cholera vibrio as a normal serum. He considered that this result was irreconcilable with Neisser and Wechsberg's explanation, as it showed that the complement was not bound to the typhoid immune-body.

Deviation of complement in the case of haemolysis has been described by Sormani (1916), who considers it due to a precipitation caused by the immune-serum on the surface of the corpuscles with a diminution of their lysability as a result. Thjøtta (1920) explains the phenomenon as resulting from the combination of antibodies with dissolved bacterial antigen to form molecular complexes which absorb complement and thus deviate it from the bacterial antibodies. In accordance with this view he found that the inhibiting action cannot be removed from an antiserum by absorbing it with a suspension of the homologous bacterium, as would be the case if the complement entered into direct union with the bactericidal immune-body. Pandit (1922-3) attributes the phenomenon to dissociation of immune-body from the bacteria ; but while this occurs to a certain extent it is not complete and we are unable to see how bactericidal action can be interfered with in this way.

Another explanation has been put forward by Schlemmer (1920). Using an antityphoid serum he finds that there are two types of immune-body (amboceptors), namely, antilipoid and antiprotein. The antilipoid immune-body in association with complement is that concerned in bacteriolysis, but the corresponding receptors can bind much more immune-body and complement than is necessary for lysis. The anti-protein immune-body when combined with bacterial protein leads to fixation of complement only, no lysis. The phenomenon of complement deviation depends upon a distribution of complement, so that each bacterium does not obtain through the antilipoid immune-body sufficient

complement for lysis. Efficient bactericidal action is thus obtained only when the complement is bound in a special way. Rodet (1924) considers that the Neisser-Wechsberg phenomenon is due to the serum possessing an antibactericidal action as well as a bactericidal. The difference in result does not depend on the quantity of immune-body but on the serum having two distinct properties, presumably carried by two antibodies. It is difficult to understand the effects of different dilutions according to this view, but one result recorded is suggestive, namely, that he has been able to modify the properties of the serum according to the mode of immunizing—according to the amount or quality of the antigen used in immunizing. The antibactericidal property is specially prominent when the highest doses tolerable are used. For details with regard to these views the original papers must be consulted.

As a result of his work on haemolysis Hyde (1928) has advanced yet another view. He considers that efficient haemolysis or bacteriolysis depends upon the components of complement being taken up in a certain order essential for the attaining of destructive effect. Excess of immune-body when present supersensitizes the antigen and this leads to the complement components being taken up in another order, protection instead of lysis resulting. The observations from which this conclusion is drawn involve the use of serum of complement-deficient guinea-pigs, the serum of these animals containing mid-piece and end-piece but lacking the third or heat-stable component.

At present a satisfactory explanation of the Neisser-Wechsberg phenomenon cannot be given. It is probably, however, to be regarded as a zone phenomenon analogous to what is seen in agglutination and precipitation (Chapters XII and XIII). It is well known that in the interaction of a protein and its antibody precipitation may be prevented by excess of antigen, and when this occurs fixation of complement also is inhibited. What occurs in the inhibition of bacteriolysis may be of corresponding nature. In connection with this subject the work of Dunlop (1928) on the fixation of guinea-pig's complement by typhoid bacilli has shown that in this animal's serum there is a substance which behaves like a natural immune-body; along with the bacilli it fixes haemolytic complement in remarkable amount and a striking zone phenomenon is present.

#### *Intensification of Bacteriolysis.*

Angerer and Hartoch (1910) have shown that treatment of bacteria with an antiserum (precipitin) to the serum containing the immune-body used in Pfeiffer's reaction intensifies the reaction. The explanation given is that the combination of precipitin with serum-protein brings a larger amount of active complement into relation with the bacteria. In their experiments they used the natural immune-body of the horse for *V. metchnikovi* and found that with the addition of a small quantity of precipitin obtained from the rabbit for horse-serum, the lysis on the

addition of complement was more rapid than in the control in which normal rabbit's serum was used. A similar phenomenon has been established in the case of haemolysis by Friedberger and Moreschi (1908), and in the case of opsonic action by Friedberger and Hartoch (1910) (Chapters IX and XI).

#### *Dissociation of Immune-Body.*

The view of Ehrlich, according to which bacteria and other cells possess a system of receptors or molecules which act as antigens, was supported and extended by Pfeiffer and his co-workers in the case of bacteriolysis. It was shown by Pfeiffer and Friedberger (1903) that just as in the case of haemolysis the bacteria may bind many times the amount of immune-body necessary for their lysis; in other words, lysis may be complete while only a fraction of the receptors are occupied by immune-body and complement. They found also that the bound immune-body becomes split off from the bacteria when lysis occurs. Thus, cholera vibrios which had taken up a great excess of immune-body and were then washed free of serum, when introduced into the peritoneal cavity of a normal guinea-pig, set free immune-body sufficient to cause the lysis of fresh vibrios in amount much greater than those sensitized with immune-body. In fact they were unable to demonstrate any distinct using up of immune-body in the process of lysis. [It may be noted that in studying the relation of immune-bodies to receptors after haemolysis, Muir (1903) found that the process did not actually set free immune-body but that the latter could be dissociated from the receptors just as in the case of immune-body attached to intact corpuscles (Chapter IX). The possibility of a similar explanation of Pfeiffer and Friedberger's results cannot be excluded.]

Pfeiffer and Friedberger's conclusions with regard to dissociation of bacteriolytic immune-body were confirmed by Bail and Tsuda (1909<sup>1</sup>) and by Spät (1910). Bail and Tsuda (1909<sup>2</sup>) also showed that the normal immune-body of the ox for cholera bacteria could be split off within the peritoneal cavity of the guinea-pig. The subject of dissociation has been more recently investigated by Huntoon and his co-workers (1921).

#### *Variety of Receptors.*

Studies on agglutination by Weil, Felix, Braun and others have shown that bacilli with peritrichous flagella have two kinds of receptors which give rise to two types of agglutinins. These are usually known as the H or flagellar and the O or somatic bacterial receptors; Braun speaks of them as 'ectoplasmic' and 'endoplasmic' respectively. This distinction is important in connection with the development of other antibodies. For details, reference must be made to Chapter XII, but it may be stated that by taking advantage of the resistance to heat and by other methods, antisera can be prepared against the two types of receptors. In the case of *B. proteus* some strains are flagellated, others non-flagellated, and this fact also has been made use of in preparing the two types of antisera. The general outcome of work on this subject has been that while

antibodies to H receptors produce agglutinins (flagellar) and in some cases precipitins, they are not, or only to a slight extent, concerned in the production of other antibodies. In the case of typhoid bacilli and other allied organisms of the same group, it is only the antisera to O receptors which are concerned in bactericidal action (Felix and Olitzki, 1926), and in accordance with this the H agglutinins in typhoid fever have little or no significance as regards the state of protection (Felix, 1924). Corresponding results have been obtained in the case of *B. tetani* and *Vibrio septique* (Felix and Robertson, 1928), and it seems probable that as a general rule bactericidal immune-bodies and complement-fixing antibodies are developed in response chiefly to somatic antigens. Lubinski (1928) reaches a like conclusion and confirms also that the antibodies to the two types of receptors vary as regards specificity. For example, in the case of *B. typhosus* and *B. enteritidis* (Gaertner) the O receptors are closely similar and thus the antiserum produced by one of these organisms protects an animal against the other. On the other hand, the H receptors are different and the corresponding agglutinins are accordingly specific, and can be used for differentiating the organisms. This is, of course, only an example of what may happen, and a general conclusion cannot yet be drawn. It has been shown recently by Braun and Nodake (1924), that it is also the somatic antigens which are chiefly concerned in the production of bacteriotropins (Chapter XI).

#### *Receptors and Virulence.*

The receptor apparatus of bacteria has also a relation to virulence. It had been shown by Van de Velde (1894) that avirulent organisms were more readily killed by normal serum than virulent, and the application of Pfeiffer's reaction gave a corresponding result. Avirulent races of the cholera vibrio, typhoid bacillus and allied organisms, undergo lysis in the peritoneum of the normal guinea-pig, but as their virulence is increased antiserum in increasing quantities is required for their dissolution. There is, in fact, a parallelism between virulence and non-susceptibility to bacteriolysis. A further result of importance established by Pfeiffer and Friedberger (1902<sup>2</sup>) is that killed virulent cholera vibrios absorb more immune-body from a cholera antiserum than do vibrios in a non-virulent state. The receptors thus either become increased in number or have an increased avidity for immune-body when the organisms become virulent. In a corresponding way killed virulent vibrios are more effective in producing active immunity than are non-virulent organisms; and, of course, it had been previously known that the high degrees of solid immunity are attained with the most virulent organisms in the living state. Pfeiffer applied such results to the explanation of virulence in one of its aspects. Thus, when virulent organisms are introduced into the tissues, the protective substance will be more quickly used up than in the case of avirulent organisms possessing less avidity. Whilst some of the virulent organisms will be killed in consequence, others will be

unaffected, there being as it were a concentration of the protective substance against only some of the invading organisms. Such a mode of action is, of course, only one factor in virulence, as formation of toxins and aggressins, alterations of the bacterial envelope and capsule formation (Chapter XI), &c., also play important parts.

*Relations of Bactericidins to Other Antibodies.*

A large amount of investigation has been carried out with regard to the relation of bactericidal immune-bodies to other antibodies, agglutinins, bacteriotropins, &c., and certain differences in the antibody response have been made out in different cases. Work on this subject has been of too extensive a nature to be reviewed fully, but, as an example, reference may be made to the detailed enquiry on the subject carried out by Henderson Smith and St. John Brooks (1912). They used typhoid vaccine for immunization and studied the curves of reaction in the case of agglutination, phagocytosis and bactericidal power. They found in the case of the first two, that the combination of antigen and reacting cell took place after the type of an adsorption process, and that with increasing doses the date at which maximum value was reached became later. On the other hand, neither of these results held with regard to bactericidal power. Further, an even more important point is that when the logarithms of the serum values were plotted against the logarithms of the doses, bactericidal action gave a curved line, whilst practically straight lines were obtained in the case of phagocytosis and agglutination. These results may be in part due to the fact that antibody formation takes place independently to the H and O antigens, but it is difficult to see how this can be applied to explain the difference between bactericidins and bacteriotropins, both of which are antibodies to O receptors.

The view widely held at an early period that there are several distinct classes of antibodies, has, however, gradually lost ground. As we have already said, the so-called types of antibodies correspond merely with types of reaction and in these reactions two or more substances are concerned—antigen *plus* antibody, and sometimes also complement. A serum *plus* antiserum reaction results in precipitation and complement fixation, and there are no grounds for believing that two antibodies are concerned, a precipitin and a complement-fixing antibody, nor is it justifiable to speak of a precipitin as the substance which precipitates. Precipitation is the result of an interaction of the antigen and antibody, and, as a matter of fact, the precipitate is chiefly formed from the antiserum. In a corresponding way, a close parallelism has been found by Weil and Felix (1920) and by Weil (1921) to exist between agglutinins and the complement-fixing antibodies, while Felix and Olitzki (1926) have come to a like conclusion with regard to the bactericidins and agglutinins developed against O receptors. It seems clear that the serum reactions are not due to different and distinct antibodies ; on the contrary the same antibody may be concerned in more than one type of reaction.

The study of the appearance of antibodies in the process of immunization shows that the reactions appear in different proportions in different cases. It must of course be recognized that an antigen is a complex, not a uniform substance, and differences in the reactions may depend upon differences in the antigen ; but it is not justifiable to explain them all in this way. Differences depend also on the animal supplying the antibodies, and even if the antigen were uniform in character it does not follow that the antibodies would also be identical. It has been shown, for example, that in the course of development of hæmolytic immune-bodies qualitative differences are met with (Chapter IX). It does not appear possible as yet to say precisely how the antibody content of a serum is to be regarded.

#### *The Relations between Different Types of Immune-Body.*

The relation between the immune-body concerned in bacteriolysis and the complement-fixing immune-body has been the subject of discussion. It is to be noted that in the ordinary complement-fixing method, what we test for is the presence or absence of complement capable of producing lysis when sensitized red corpuscles are added. It is evident, however, that instead of sensitized corpuscles we may use bacteria *plus* immune-body and observe whether or not free complement is present by the effect on the bacteria ; the bacteria in question must, of course, be sensitive to the bactericidal action of the serum. We cannot say *a priori* whether the results will be the same in the two cases—that is, that hæmolysis and bactericidal action will correspond. Complement concerned in opsonic action (Chapter XI) is also brought into union with the bacteria through the medium of immune-body, and the fixing of opsonic complement can be tested in a similar way ; this, however, does not concern us here. It is sufficient to keep in view that complement can be recognized only by its effects—bacteriolytic, hæmolytic or opsonic action. Complement is fixed by various combinations of antigen *plus* antibody—bacteria *plus* immune-body, red corpuscles *plus* immune-body, proteins *plus* antibody (precipitin). On investigation, however, it is found that complement is not absorbed in a uniform manner by these different combinations. For instance, red corpuscles treated with immune-body absorb practically no complement at 0° C. under ordinary conditions. [When immune-body is in great excess, however, some union of complement and lysis may occur (Haendel, 1908).] On the other hand, a serum precipitate (protein *plus* immune-body) fixes complement at 0° C. (Dean, 1916-17). In fact the latter observer found that more complement is fixed at 0° C. than at 37° C., though the combination of complement occurs more rapidly at the higher temperature. A certain amount of opsonic complement also is fixed at 0° C. (Chapter XI).

With these facts in view we may consider the behaviour of antisera which are both bacteriolytic and complement-fixing. Neufeld and Haendel (1908) found that in the case of anticholera sera the properties differ in certain points. Using an anticholera serum with little complement-fixing

property, they observed the absorption of complement as tested by haemolytic and bactericidal action. They found that no complement was fixed at 0° C. and only the bactericidal complement at 37° C. On the other hand, by the use of other strains, sera possessing more complement-fixing properties and with lower bactericidal action than that of the previous serum were obtained, and with them Neufeld and Haendel found that at 0° C. haemolytic complement was fixed whereas bactericidal complement was not. At 37° C. both bactericidal and haemolytic complements were fixed.

It will be seen from the above account that complement concerned in bactericidal action may be absorbed whereas there is little or no absorption of haemolytic complement. In this respect we may speak for descriptive purposes of a bactericidal immune-body as contrasted with a complement-fixing immune-body. This result of Neufeld and Haendel corresponds with what was observed by Muir and Browning (1909) in the case of normal bactericidal action. The latter observers found that when a normal serum was absorbed by a suspension of a given bacterium, the first effect was the disappearance of bactericidal action for that bacterium. Absorption with a larger amount of the bacteria in question removed the bactericidal action for other bacteria, whilst the haemolytic complement was scarcely affected. More energetic treatment still led to absorption of haemolytic complement. They accordingly concluded that a moiety of complement concerned in bactericidal action is first removed by bacteria, and spoke of this as 'bacteriophilic complement'. It has been established by these and other results, notably in haemolysis, that complement is not a uniform substance, but may be regarded as consisting of moieties with different properties.

As regards the *constitution* of complement, as opposed to modes of action, no difference has been established between bactericidal and haemolytic complements. Splitting of complement (Chapter X), for instance, has given fractions which are concerned in the same way in the two types of lysis (Braun, 1911, and Boehncke, 1912). It has recently been shown by Gordon, Whitehead and Wormall (1926) that there is a fourth component of complement which is relatively thermo-stable, resisting a temperature of 56° C. for half an hour, and which is destroyed in almost a specific way by ammonia. On testing the relation of this component to lysis it was found that it was concerned in the same way in both bactericidal action and haemolysis (Gordon and Wormall, 1928). On the other hand, it was not essential to the opsonic action of complement (Chapter XI).

We may emphasize here what has been referred to above, namely, that variations in the bactericidal action of normal sera do not depend upon combining affinities, but are due to the relative sensitiveness of organisms to complement—some are killed whilst others are not. Steinhardt (1905) studied this question in connection with the deterioration of bactericidal action towards various bacteria and found that the differences observed depended on a quantitative and not on a qualitative

basis. The bactericidal action fell first in the case of organisms most difficult to kill. She laid stress on variations in the toxic action of complement, as had previously been done in the case of haemolysis by Muir and Browning (1904).

#### *Bacteriolysis in Relation to Antitoxic Action.*

It was clearly established by work of Pfeiffer and others who followed him that in many instances potent bactericidal sera might have no more action on the toxic properties of the dead organisms (endotoxins) than normal sera had. He, however, did not state that no antitoxins could be produced to endotoxins. As a matter of fact, antitoxins have been obtained to the toxins of such organisms (see Metchnikoff, Roux and Taurelli-Salimbeni, 1896; Macfadyen, 1906; Brau and Denier, 1906; Kraus and Russ, 1908). There is also the well-known example of the El Tor cholera vibrio which produces a powerful exotoxin; in this case an antitoxin as well as a bactericidin can be developed on immunization. The fact remains, however, that antitoxic action usually plays little part in cases where bactericidins are concerned and in many instances cannot be demonstrated. The important point is, as insisted upon by Pfeiffer, to recognize the two distinct modes of action of antisera—that leading to death of bacteria and that leading to neutralization of toxin. To what degree each is present must be determined in the case of any particular organism (see Huebschmann, 1928).

An important point in connection with antitoxic action is brought forward by Pfeiffer and Bessau (1910) in the case of an antityphoid serum which had a certain degree of neutralizing action on the toxic action of dead typhoid bacilli, with, at the same time, a strong bacteriolytic action. They found on investigation that such a serum did not behave as an ordinary antitoxin. For example, while a fatal result might be prevented by its use it was not possible to avoid entirely the toxic effect of the dead organisms. Its action, moreover, did not follow the law of multiple proportions. Further, they found that a large quantity of serum might have a less favourable result than a smaller quantity (this suggesting something like deviation of complement), and that the action of the serum was intensified by the artificial production of leucocytosis in the peritoneal cavity. They concluded that the results could not be explained on a simple antitoxic basis and they put forward the view that the favourable effect of the serum was due to destructive or disintegrative action on the endotoxin, this resulting from the action of complement along with immune-body, and thus being of the same nature as bacteriolysis itself. Bacteriolysis occurs, in fact, at an early stage; neutralization of endotoxin, or, as it might be called, *detoxication* takes place later and may not be apparent. The nature of endotoxins is outside the present article, but we mention these results as they show the possibility of an additional effect of immune-body along with complement. It may be added that in a recent paper Eisler (1928) describes the formation of antitoxins to endotoxins or,

as he calls them, toxoplasmins. Like Pfeiffer and Bessau, he states that the antitoxic action in such cases does not follow the law of multiple proportions.

#### *Normal Bactericidal Action.*

This subject has been dealt with in Chapter II and we shall consider here only the constitution of the substances concerned. The fact that immune-body and complement are concerned in the bactericidal action of an antiserum naturally raised the question whether a similar dual constitution is concerned in the bactericidal action of normal serum ; and it was established in various instances that this is the case. This result has been obtained alike by Pfeiffer's reaction, by the Bordet-Gengou method of complement-fixation and by the study of direct bactericidal action. Pfeiffer (1895<sup>2</sup>) showed that normal goat's serum inactivated at 55° C. was activated within the peritoneal cavity of the guinea-pig and caused bacteriolysis. Later, in association with Friedberger (1902<sup>1</sup>), he found that anti-immune-body against a cholera antiserum from the goat acted also on the normal immune-body of that animal. Moxter (1899) demonstrated *in vitro* a normal immune-body ; he showed that inactivated guinea-pig's serum was activable by the fresh peritoneal fluid of that animal. Again, Wechsberg (1902) found that guinea-pig's complement along with inactivated normal rabbit's serum killed the typhoid bacillus, but this result was not obtained when rabbit's complement was used. [Rabbit's whole serum is, however, actively bactericidal (Felix and Olitzki, 1926).] Using the complement-fixing method, Malvoz (1902) showed that in normal dog's serum there is an immune-body for the anthrax bacillus which leads to the fixation of the complement of rabbit, guinea-pig and rat ; whilst no such immune-body in the serum of the guinea-pig or ox can be demonstrated. The normal serum of the ox contains a considerable amount of immune-body for the cholera vibrio (Bail, 1905<sup>2</sup>). It was shown by Muir and Browning (1909) that treatment of a fresh serum with a particular bacterium removes first the bactericidal action against that bacterium, later that for other bacteria, and later still haemolytic complement. The first result may well depend upon a specific immune-body for the bacterium used for absorption, but the second result cannot be so explained (p. 281). The action of a normal serum has been especially worked out in the case of haemolysis, and in this case there is no doubt that, as a rule at least, natural immune-body along with complement is concerned in the lytic process. Some writers, e.g. Bordet (1920), consider that an immune-body is concerned generally in the bactericidal action of a normal serum ; on the other hand, Gruber (1901) maintained that attenuated bacteria might be killed by the direct action of complement. It may be noted that Wright and Windsor (1902), in their bactericidal studies, found no evidence of an immune-body in the action of a normal serum on the typhoid bacillus and the cholera vibrio ; treatment of the serum with either organism removed the bactericidal action on the other. They found also that the

addition of the filtrate of a macerated culture removed the bactericidal action, just as dead bacilli did ; the filtrate of a fresh culture, on the other hand, had no such effect. Buxton (1905) obtained a somewhat different result ; he found that absorption of normal serum with the typhoid bacillus removed the bactericidal action for the cholera vibrio, but that the converse did not obtain. Accordingly, although the action of natural immune-bodies concerned in bactericidal action has been demonstrated by the methods in a large number of instances, it appears scarcely justifiable to infer that this holds universally. The case is different from that of haemolysis, because complement is not fixed by red corpuscles unless they are sensitized by immune-body, whereas bacteria, like various organic particles, absorb complement directly, and it may be that the complement thus combined may sometimes have bactericidal effect. The recent work of Gordon and Wormall (1928) and Gordon, Whitehead and Wormall (1929) on bactericidal action suggests that in the treatment of fresh serum with large amounts of dead bacteria (unsensitized) the fourth component of complement, necessary for bactericidal action and haemolysis, is not readily removed. They showed clearly that a natural immune-body was concerned in the bactericidal action of normal guinea-pig's serum on *B. dysenteriae* (Flexner).

#### *Bactericidal Action apart from Complement.*

Although no satisfactory evidence has been forthcoming that complement is formed by leucocytes, it is now thoroughly established that these cells are the source of other bactericidal substances which are usually known as *leukins*. The importance of leucocytes in bactericidal action was shown by Buchner (1894) and Hahn (1895), but the differences between the bactericidal substances contained in them and the serum alexins was first clearly brought out by Schattenfroh (1897), and his results have been fully confirmed. The methods employed in this enquiry have been first of all to produce an exudate rich in polymorphs by aleuronat or other means, and then to treat the exudate by such methods as freezing and thawing, aided by mechanical disintegration, or treatment with weak acids or alkalis, or digesting in saline at various temperatures, &c. (Schattenfroh, 1897 ; Kling, 1910 ; Zinsser, 1910 ; Blum, 1922 ; Haussmann, 1925). It has been conclusively shown that the leukins thus obtained differ from complement in various respects, such as the following. The leukins are relatively stable, being as a rule unaffected by a temperature of 55 to 60° C. for half an hour, while some of them require a much higher temperature, even 75° C. for their destruction. In the case of any one animal the leukins are bactericidal towards different organisms from those killed by the serum, and the former have as a rule a wider bactericidal action than complement, this being exerted against such bacteria as *B. anthracis*, *Pneumococcus*, *Streptococcus*, *M. tetragenus*, &c., as well as many saprophytes. Their activity, however, is found to vary greatly in the case of different animals. Unlike complement again,

their action is little affected by salt concentration and is not removed by dialysis against water (Schattenfroh, 1897). Their action, moreover, is a direct one, there being no evidence as a rule of an intermediary like immune-body. Weil (1909), however, describes a process like reactivation of serum by leukins, and Blum (1922) records a similar phenomenon. Another important point is that leukins do not lead to the lysis of sensitized red corpuscles. Most writers consider that the leukins are set free on the disintegration of the cells, but Schneider (1909, 1911) favours the view that there is a real secretion and ascribes the bactericidal action of tissue fluid to this process. Actual secretion of leukins, however, can hardly take place to any extent, otherwise there would not be the striking differences between the extracts of leucocytes and the serum bactericidins as regards the organisms on which they act in any given case (see Knorr, 1929). A modified view of the action of leucocytic bactericidins has been put forward by Weil (1911). He found in certain cases that such substances are set free from leucocytes not spontaneously but only under the stimulus exerted by bacteria in their presence, and he found also by means of combination methods that the bacteria in question had an affinity for the bactericidal substances. He applied the term 'aphagozidie' to this type of bactericidal action, which occurs without phagocytosis and apart from spontaneous secretion. True lysis of the bacteria studied was observed to result from this mode of action.

While thermo-stable bactericidins have been demonstrated in serum, their exact relationships to leukins extracted from leucocytes is, however, imperfectly known. It has long been recognized that the normal serum of certain animals kills the anthrax bacillus, but the hope entertained at an early stage that in this way the natural immunity of different animals against anthrax could be explained was not realized. The serum of the relatively susceptible rabbit is strongly bactericidal while the sera of the immune fowl and dog have little action. It was also established that complement is not concerned in bactericidal action against *B. anthracis*, *B. subtilis*, &c.; more recently it has been shown that the same holds with regard to various anaerobes. The bactericidal action of normal serum against these organisms is not destroyed by heating at 55° C. for more than half an hour; and it is not removed by shaking with ether or by diluting with water, as is the case with bactericidal action in which complement is concerned. The source of the bactericidal substances appears to be mainly in the leucocytes and the term endolysins has been applied to such substances. According to Gruber and Futaki (1907) plasma free from leucocytes is not bactericidal to the anthrax bacillus, whereas serum or plasma along with leucocytes may have distinct action.

It has also been found that the platelets are another source of bactericidal substances, these being known as *plakins*. Gruber and Futaki found that plakins, while usually anthracocidal, have no such effect when the bacilli are encapsulated. Great variations in the amounts of plakins have been found in different animals.

But even with those facts established the explanation of natural immunity against such organisms is still a complicated subject (see Ledingham, 1922). It is, however, generally recognized that the leucocytes play a very important part, and that they act either by setting free bactericidal substances or by ingesting the organisms and then killing them. Anything interfering with the activity of the leucocytes in either of these directions will break down the defences and thus aid infection. There have now been accumulated various facts which go to show that the anthrax and other bacilli in the body fluids form substances known as aggressins which interfere with the phagocytic activity of the leucocytes and also with their response to the stimulus to set free bactericidal substances. And it is also reasonable to conclude from observations made that the leucocytes of different animals vary in their susceptibility to aggressins. This varying susceptibility, of course, cannot be explained, but it is an example of what commonly occurs. There is also evidence that when the leucocytes possess this insusceptibility to injury by aggressins, they act either by setting free leukins or by intracellular destruction. It may be noted that in some instances the leucocytes adhere to the bacilli without ingesting them and still bring about their death—the so-called 'contact killing' (Gruber and Futaki, 1907). In the production of active immunity against anthrax to be presently considered, there is considerable evidence that the presence of immunity is due to anti-aggressins developed in the process of immunization and that the possibility of producing passive immunity is due to the transference of these substances.

*$\beta$ -Lysins* (Pettersson). To the above account we may add some of the main results obtained by Pettersson (1924-8) with regard to relatively thermo-stable substances which he calls  $\beta$ -lysins. These substances are more stable than ordinary complement, are not removed from the serum by shaking with ether nor destroyed on diluting with water. Pettersson finds that the substances concerned are composed of two parts, an activating substance and an activable substance; the former is the more labile but is less so than complement and requires for its inactivation a temperature of about 63° C. for half an hour. The activable substance is still more thermo-stable. The activating substance is as a rule present in much larger quantities in the serum than the activable. In order to reactivate a serum inactivated by heat a tenth of its volume of fresh serum is often sufficient, the fresh serum added being without bactericidal effect by itself. Sera non-bactericidal towards bacteria often contain activable substance, as is shown on the addition of a small quantity of fresh serum. The activating substance in addition to being present in plasma is found also in the leucocytes and platelets. Less is known about the activable substance, but it only unites with bacteria in the presence of the activator. Thus it cannot be absorbed from an inactivated serum by treatment with the bacteria. On the other hand it is fixed directly by tissue cells. Pettersson considers that the  $\beta$ -lysins are not the same as

the leukins or endolysins of leucocytes described by others. Among the organisms against which  $\beta$ -lysins have been found to be active may be mentioned *B. anthracis*, *B. subtilis*, *B. tenuis*, *B. murisepticus*, various anaerobes, &c. The relations of the various thermo-stable bactericidins contained within leucocytes or present in the serum are much in need of further elucidation.

The fluids of the body may exert bactericidal effect in still other ways than those described. There is, for instance, the interesting action of lysozyme first discovered by Fleming (1922), and described on p. 42. Another example is the bactericidal action of cerebrospinal fluid on certain bacteria, recently described by Ikegami (1926). In this action neither complement nor leucocytes play a part and the substance concerned is relatively thermo-stable. None of these substances, however, undergo increase during the process of immunization, such an increase being confined, as already stated, to antibodies.

### General Considerations.

It has already been pointed out that the production of antibodies in immunity is merely an example of the response to the parenteral introduction of antigens in general. We have now to consider which antibodies have an actual relation to the state of protection and to what extent their presence can be regarded as explanatory of the latter. The controversy between the humoral and cellular theories of immunity is now a thing of the past. It is recognized, on the one hand, that immunity in certain cases is due to the presence of antibodies in the blood. On the other hand, it is no longer a matter of dispute that antibodies are the products of cellular activity, that the cells which have taken part in the antibody response are functionally altered in consequence, and lastly that immunity may exist after antibodies have disappeared from the blood.

In any given case it may be a matter of difficulty, or it may be impossible, to say to what extent the immunity depends upon antibodies and to what extent upon the underlying cellular changes. We may, however, in the first place adopt as a working principle that whenever passive immunity can be produced, this is due to the transference of antibodies in the serum. In some instances, e.g. anti-anthrax serum (p. 291), the nature of the antibodies is still a subject of question, but there is no evidence that an antiserum exerts a specific stimulus on the cells of the animal injected or acts in any other way than by the antibodies which it contains. Actual protection by antisera is the all-important matter. The degree of protection can be quantitatively estimated in terms of the amount of serum required to produce a given result under given conditions, and this can then be compared with the content of the serum in different types of antibodies. Unless this is done it is unjustifiable to infer anything with regard to the protective power of the serum from the concentration of a given type of antibody as tested by in-vitro

reactions. We have thus to consider the significance of antibodies present in the serum in relation to their protective value and the state of immunity to which they give rise.

In the first place, immune-bodies which lead to direct bactericidal action, with or without bacteriolysis, are of high importance in immunity both active and passive. The work of Pfeiffer and his co-workers established this in the case of the cholera vibrio, typhoid bacillus and allied organisms, and a close parallelism was shown to exist between the occurrence of bacteriolysis, both *in vivo* and *in vitro*, and the state of immunity. And the organisms which are readily susceptible to bacteriolytic action have been shown also to undergo rapid dissolution when introduced into the blood of an immune animal. Bacteriolysis may thus be regarded in a sense as the extreme example of the destructive effect on bacteria produced by an antiserum. As has been stated above, however, the number of bacterial species which are susceptible to this direct action of the serum are comparatively few, and thus only a small part of immunity can be explained on the basis of bactericidal action of the fluids apart from phagocytosis. It is customary to divide organisms into those which are susceptible and those which are non-susceptible to bactericidal action, but the line of demarcation cannot be regarded as a strict one, as we may justifiably consider that, short of actually killing organisms, sera possess all degrees of inhibiting action which may be of importance.

A much more widely applicable, and, in this respect, more important means of defence than bactericidal action is supplied by the opsonic antibodies—bacteriotropins and opsonic immune-bodies (Chapter X). That the serum of an immunized animal usually contains antibodies which sensitize the corresponding bacteria to phagocytosis is a fact of far-reaching importance. Many antisera without bactericidal action contain bacteriotropins in abundance, and even when bactericidal action is present organisms which escape being directly killed can often be seen to be ingested by leucocytes and other cells, especially those of the reticuloendothelial system. Further, direct microscopic observation shows that phagocytosis is often followed by destruction of the organisms, and in certain cases it has been demonstrated *in vitro* that an antiserum *plus* leucocytes may cause death of the bacteria while the serum alone has no such effect (p. 372). In many instances little or no digestion of the organisms is seen in experiments *in vitro* but all degrees of morphological change up to complete dissolution within leucocytes can be followed in the living body, e.g. in the case of such organisms as streptococci, staphylococci, &c. The question as to the relation between extracellular and intracellular destruction of organisms is discussed later (p. 374), and the conclusion is reached that the destruction of the organisms in the two situations does not depend upon the same agencies. There is no satisfactory evidence that intracellular digestion is due to complement and that the opsonic antibodies act by leading to the union of complement after the bacteria have been ingested by the cells. The evidence rather shows that

the antibodies bring about ingestion of the organisms by phagocytes and that within the latter the organisms are destroyed by leukins and other substances different in nature from complement. There is also the possibility that the process of intracellular digestion leads to the destruction of endotoxins and thus the body is protected against their action. Whilst these statements may be made, it must, however, be recognized that phagocytosis in itself does not mean protection. Even in fatal infections the phenomenon may be observed, and active ingestion of bacteria may be seen in chronic infections of progressive nature, e.g. tuberculosis and leprosy. In gonococcal infection also active phagocytosis, without immunity and without cure, is a prominent feature ; indeed the intracellular gonococci often show no evidence of degeneration and a sort of symbiosis appears to be established. Other examples of like nature might be adduced, but nevertheless the fact remains that the intracellular position is the one most favourable for the destruction of the organisms, and thus the antibodies in the serum which lead to its occurrence are of great significance in both active and passive immunity. It need hardly be pointed out that the recognition of such antibodies has been the means of linking up the properties of serum, insisted on by the supporters of the humoral theory, with the cellular means of defence. The bacteria on their part do not remain passive in the tissues but often acquire increased virulence, which is not infrequently indicated by decreased susceptibility to phagocytosis.

Agglutination and bacterio-precipitation may be placed together as there is no evidence that either type of action by itself leads to any injurious effect on the bacteria and thus to immunity. The development of agglutinins often goes *pari passu* with that of protective substances, and when such association can be relied upon, the estimation of agglutinins, which can be so readily carried out, comes to be of importance. But such a relationship must be determined in any given case of infection. Since the O or somatic and the H or flagellar agglutinins have come to be recognized, it has been shown that the former are specially related to the state of immunity. More extended observations on this subject are required before a definite statement can be made as to what this relationship actually is.

The definite relationship of all these antibodies to the corresponding antigens can readily be shown by treating or absorbing the antisera by the homologous bacteria ; by such treatment the antibodies, owing to their combining affinities, can be removed from the serum. In the case of certain sera, of which anti-anthrax serum has been most fully studied, this result is not obtained, yet the antiserum may be protective, and this property is due to antibodies. The corresponding antigens are not present in the bacteria as met with in culture and the antibodies appear to be developed in response to antigens which are formed only by the organisms when growing in the tissues of the body. They are thus possibly of the nature of anti-aggressins to which reference is made below (p. 290).

While anthrax has been specially studied it is recognized that protection against various other infections is produced much more efficiently by living than by dead organisms (Chapter III). Theoretically the explanation of this might rest on a quantitative or a qualitative basis. It might, on the one hand, depend on antigens being produced by living organisms gradually and in amounts which are specially efficient in leading to antibody production—in a manner which cannot be satisfactorily imitated by the injection of dead vaccines. On the other hand, it might be due to the fact that the living organisms when growing in the tissues produce substances of the aggressin class, and that antibodies are developed against these. It is at least likely that if there is a real anti-aggressin immunity, this is not confined to the organisms which have been specially studied but obtains generally in varying degree. The superior efficiency of the living organisms as immunizing agents in certain cases is, however, a subject which requires further elucidation. What has been said concerns mainly the means by which immunity rests upon enhanced means of destroying the bacteria, but that may be associated with immunity to their toxins to varying extent. When these are neutralized in true infections the various means which the body possesses for destroying bacteria will necessarily be allowed a freer action.

We have considered immunity so far as serological changes in the immune animal are concerned, but the whole subject cannot be explained in this way. It is known that important changes occur in the cells of the body as a result of the functional activity in producing antibodies or apart from such function. The evidence for the occurrence of such cellular alterations is dealt with elsewhere, but may be said to fall under three main headings. In the first place, it is known that after antibody response has occurred the functional capacity of the cells, so far as antibody protection is concerned, has undergone alteration, and that immunity may persist after antibodies have disappeared from the blood. In the second place, in the state of anaphylaxis which also depends upon antibody production, the tissues of the sensitized animal respond to antigen in a different way from those of a normal animal. And lastly, local immunity may be produced by bacterial vaccines without there being any evidence of antibodies being concerned.

#### **Anti-aggressins.**

By C. H. BROWNING.

The existence of a particular type of antigen, to which Bail (1905<sup>1</sup>) applied Kruse's name of 'aggressin', has been the subject of a considerable amount of investigation and also controversy (see Sauerbeck, 1907). In the present connection, the existence of aggressins rests, especially on the evidence that (1) in the case of infections with certain highly virulent organisms, which typically show a septicæmic course, e.g. anthrax (Bail, 1904) and fowl-cholera (Weil, 1905), the oedema fluid or serous exudate

from infected animals, when freed from organisms and sterilized, acts in some cases as a good immunizing agent ; and that (2) passive immunity is conferred by the serum of animals which have been rendered actively immune by injections of such aggressins. The marked immunizing property of aggressin-containing exudates contrasts with the very slight degree of immunizing power shown by ordinary killed cultures of these organisms. Hence Bail held that the aggressins are formed exclusively, or, at least, mainly *in vivo* (see also Bail and Weil, 1911). As regards obtaining satisfactory specimens of aggressin, it is to be noted that in the case of the fowl-cholera bacillus Weil inoculated rabbits intrapleurally, then after the death of the animals drew off the pleural exudate, which was turbid from the presence of organisms but almost devoid of cells, and added 0·5 per cent. phenol ; the fluid was cleared by filtering through paper and centrifuging and finally was heated at 44° C. for three hours. It was found to be important not to exceed the latter temperature, as otherwise the aggressin tended to be damaged. The sterility of the fluid obtained required to be ascertained by subculture. The conditions necessary for obtaining exudates rich in aggressins appear, however, to vary according to the organisms (Bail and Weil, 1911).

It has also been stated by Bail (1905<sup>1</sup>) and his co-workers that aggressins are formed *in vivo* by other organisms, such as *B. typhosus* and *V. cholerae* ; but it is difficult to determine in how far these so-called aggressins differ from products of the organisms which may be extracted from cultures *in vitro* by various means, e.g. by macerating with serum, saline or water and which include endotoxins (Wassermann and Citron, 1905 ; see also Bürgers and Hösch, 1909). Further, according to Citron (1906) such watery extracts of cultures of the bacillus of swine-plague possess efficient immunizing properties similar to those of aggressin obtained according to Weil's procedure. Also, the serum of animals immunized with the watery extract conferred passive immunity.

According to Bail (1904, 1905<sup>1</sup>), anti-aggressins are produced by injecting into suitable animals aggressin-containing exudates. Thus, rabbits may be readily immunized against *B. anthracis* by subcutaneous injections of the sterilized oedema fluid of a rabbit which has died from anthrax infection. The antiserum developed by such means confers protection when injected parenterally into suitable animals. The mechanism of this action has long been obscure, since anti-anthrax serum was known not to contain effective antibacterial reaction-bodies and the bacilli do not form any demonstrable toxin. Recently, the problem has been investigated anew by Pettersson (1928<sup>2</sup>). He has confirmed the absence from the antiserum of bactericidal immune-bodies demonstrable *in vitro*. Also, the antiserum does not appear to be readily deprived of its protective action by treatment in the test-tube with living or killed anthrax bacilli—although addition of aggressin-containing oedema fluid inhibits its action (Matsumoto, 1924). Further, the presence of immune bacteriotropins cannot be demonstrated either *in vitro* or *in vivo*. The absence of such

action in the living body was shown by the following method. Subcutaneous injections were made into rabbits of mixtures of leucocytes and anthrax bacilli along with normal rabbit-serum on one side and with immune-serum on the other. When the sites at which the injections had been made were examined some hours later very little phagocytosis was seen in either case. On the other hand, the accumulation of leucocytes which occurs at the site of subcutaneous inoculation with virulent bacilli in an immune animal, in contrast to the absence of such a collection in the susceptible individual, is explained by the following experiment. Concentrated anthrax oedema fluid was mixed with a virulent culture of *B. anthracis* and divided into two portions; to one of these normal rabbit's serum was added, to the other immune-serum. The mixtures were injected subcutaneously into a rabbit, one on each side. Two days later it was found that at the side on which the mixture containing immune-serum had been injected there was an abundant collection of polymorphonuclear leucocytes, whereas on the other practically nothing was seen. The results support Bail's original view that the aggressin contained in the oedema fluid has the property of antagonizing the normal protective mechanism, and that the antiserum neutralizes this effect. The leucocytes were originally thought to be the chief protective agents, but in the case of *B. anthracis*, according to Singer's (1925) observations, the protective function is exercised mainly by the cells of the reticuloendothelial system, the histiocytes. Thus, the actively or passively immune animal would differ from the normal individual owing to the greater and more prolonged phagocytic activity of the histiocytes in the former than in the latter.

## REFERENCES.

ANGERER, C. & HARTOCH, O., 1910, *Z. ImmunForsch.*, Tl. I, Orig., **4**, 210.  
 ARMSTRONG, R. R., 1925, *Proc. Roy. Soc., B*, **98**, 525.  
 BAIL, O., 1904, *Zbl. Bakter.*, Abt. I, Orig., **36**, 266, 397; 1905<sup>1</sup>, *Arch. Hyg.*, Berl., **52**, 272; 1905<sup>2</sup>, *Deuts. med. Wschr.*, **31**, 1471, 1788.  
 BAIL, O. & ROTKY, K., 1913, *Z. ImmunForsch.*, Tl. I, Orig., **17**, 378.  
 BAIL, O. & TSUDA, K., 1909<sup>1</sup>, *Zbl. Bakter.*, Abt. I, Orig., **48**, 194; 1909<sup>2</sup>, *Z. ImmunForsch.*, Tl. I, Orig., **1**, 772.  
 BAIL, O. & WEIL, E., 1911, *Arch. Hyg.*, Berl., **73**, 218.  
 BEHRING & NISSEN, F., 1890, *Z. Hyg.*, **8**, 412.  
 BEZZOLA, C., 1909, *Zbl. Bakter.*, Abt. I, Orig., **50**, 541.  
 BLUM, K., 1922, *Arch. Hyg.*, Berl., **91**, 373.  
 BOEHNCKE, K. E., 1912, *Z. ImmunForsch.*, Tl. I, Orig., **13**, 240.  
 BORDET, J., 1895, *Ann. Inst. Pasteur*, **9**, 462; 1896, *ibid.*, **10**, 104, 193; 1898, *ibid.*, **12**, 688; 1920, *Traité de l'immunité*, Paris, 389.  
 BORDET, J. & GAY, F. P., 1908, *Ann. Inst. Pasteur*, **22**, 625.  
 BRAU & DENIER, 1906, *Ann. Inst. Pasteur*, **20**, 578.  
 BRAUN, H., 1911, *Z. ImmunForsch.*, Tl. I, Orig., **9**, 665.  
 BRAUN, H. & NODAKE, R., 1924, *Zbl. Bakter.*, Abt. I, Orig., **92**, 429.  
 BUCHNER, H., 1890, *Arch. Hyg.*, Berl., **10**, 84, 101, 121, 149; 1894, *Münch. med. Wschr.*, **41**, 469.  
 BÜRGERS, TH. J., 1910, *Z. ImmunForsch.*, Tl. I, Orig., **5**, 638.  
 BÜRGERS & HÖSCH, 1909, *Z. ImmunForsch.*, Tl. I, Orig., **2**, 31.  
 BUXTON, B. H., 1904-5, *J. Med. Res.*, **18**, 305, 431, 461.  
 CITRON, J., 1906, *Zbl. Bakter.*, Abt. I, Orig., **40**, 153.

DEAN, H. R., 1916-17, *J. Path. Bact.*, **21**, 193.  
 DUNLOP, E. M., 1928, *J. Path. Bact.*, **31**, 769.  
 EISLER, M., 1928, *Z. ImmunForsch.*, **56**, 209.  
 FELIX, A., 1924, *J. Immunol.*, **9**, 115.  
 FELIX, A. & OLITZKI, L., 1926, *J. Immunol.*, **11**, 31; 1929, *Brit. J. Exp. Path.*, **10**, 26.  
 FELIX, A. & ROBERTSON, M., 1928, *Brit. J. Exp. Path.*, **9**, 6.  
 FLEMING, A., 1922, *Proc. Roy. Soc.*, B, **93**, 306.  
 FRAENKEL, C. & SOBERNHEIM, 1894, *Hyg. Rdsch.*, **4**, 97, 145.  
 FRIEDBERGER, E. & HARTOCH, O., 1910, *Z. ImmunForsch.*, Tl. I, Orig., **4**, 218.  
 FRIEDBERGER, E. & MORESCHI, C., 1908, *Zbl. Bakt.*, Abt. I, Orig., **45**, 346.  
 GAY, F. P., 1905, *Ann. Inst. Pasteur*, **19**, 593.  
 GENGOU, O., 1901, *Ann. Inst. Pasteur*, **15**, 68, 232.  
 GORDON, J. & WORMALL, A., 1928, *J. Path. Bact.*, **31**, 753.  
 GORDON, J., WHITEHEAD, H. R. & WORMALL, A., 1926, *Biochem. J.*, **20**, 1028, 1044; 1929, *J. Path. Bact.*, **32**, 57.  
 GÓZONYI, L., 1913, *Z. ImmunForsch.*, Tl. I, Orig., **19**, 172.  
 GRUBER, M., 1901, *Münch. med. Wschr.*, **48**, 1965.  
 GRUBER, M. & FUTAKI, K., 1907, *Deuts. med. Wschr.*, **33**, 1588.  
 HÄNDEL, 1908, *Arb. GesundhAmt.*, Berl., **28**, 511.  
 HAHN, M., 1895, *Arch. Hyg.*, Berl., **25**, 105.  
 HAHN, M. & NEU, H., 1920, *Z. ImmunForsch.*, Tl. I, Orig., **29**, 349.  
 HAUSSMANN, A., 1925, *Arch. Hyg.*, Berl., **95**, 69.  
 HUEBSCHMANN, P., 1928, *Zbl. Bakt.*, Abt. I, Orig., **108**, 87.  
 HUNTOON, F. M., 1921, *J. Immunol.*, **6**, 117.  
 HYDE, R. R., 1928, *Amer. J. Hyg.*, **8**, 730.  
 IKEGAMI, Y., 1926, *Z. ImmunForsch.*, **46**, 522.  
 KLING, C. A., 1910, *Z. ImmunForsch.*, Tl. I, Orig., **7**, 1.  
 KNORR, M., 1929, *Handb. d. path. Mikroorg.*, hrsg. Kolle, Kraus u. Uhlenhuth, Jena, **2**, 663.  
 KRAUS, R., 1928, *Zbl. Bakt.*, Abt. I, Orig., **108**, 163.  
 KRAUS, R. & RUSS, V. K., 1908, *Zbl. Bakt.*, Abt. I, Orig., **45**, 332, 417.  
 LAMBOTTE, U., 1903, *Zbl. Bakt.*, Abt. I, Orig., **34**, 453.  
 LECLAINCHE, E. & MOREL, CH., 1901, *Ann. Inst. Pasteur*, **15**, 1.  
 LEDINGHAM, J. C. G., 1922, *Lancet*, Lond., ii, 898.  
 LEVIN, E. I., 1909, *Z. ImmunForsch.*, Tl. I, Orig., **1**, 3.  
 LIPPmann, 1916, *Z. ImmunForsch.*, Tl. I, Orig., **24**, 107.  
 LIPSTEIN, A., 1902, *Zbl. Bakt.*, Abt. I, Orig., **31**, 460.  
 LÖFFLER, F. & ABEL, R., 1896, *Zbl. Bakt.*, Abt. I, Orig., **19**, 51.  
 LUBINSKI, H., 1928, *Zbl. Bakt.*, Abt. I, Orig., **108**, 200.  
 MACFADYEN, A., 1906, *Proc. Roy. Soc.*, B, **77**, 548.  
 MALVOZ, E., 1902, *Ann. Inst. Pasteur*, **16**, 625.  
 MATSUMOTO, T., 1924, *Z. ImmunForsch.*, **40**, 402.  
 METCHNIKOFF, E., 1893, *Ann. Inst. Pasteur*, **7**, 403; 1895, *ibid.*, **9**, 433.  
 METCHNIKOFF, E., ROUX, E. & TAURELLI-SALIMBENI, 1896, *Ann. Inst. Pasteur*, **10**, 257.  
 MORRISON, L. F., 1922, *J. Immunol.*, **7**, 435.  
 MOXTER, 1899, *Zbl. Bakt.*, Abt. I, Orig., **26**, 344.  
 MUIR, R., 1903, *Lancet*, Lond., ii, 446.  
 MUIR, R. & BROWNING, C. H., 1904, *Proc. Roy. Soc.*, B, **74**, 298; 1909, *J. Path. Bact.*, **13**, 76.  
 NEISSER, M. & WECHSBERG, F., 1901, *Münch. med. Wschr.*, **48**, 697.  
 NEUFELD, F. & HÄNDEL, 1908, *Arb. GesundhAmt.*, Berl., **28**, 198.  
 NUTTALL, G. H. F., 1888, *Z. Hyg.*, **4**, 353.  
 PANDIT, C. G., 1922-3, *J. Hyg.*, Camb., **21**, 406.  
 PETRIE, G. F., 1904, *J. Path. Bact.*, **9**, 130.  
 PETTERSSON, A., 1924, *Z. ImmunForsch.*, **40**, 43; 1926, *ibid.*, **48**, 233; 1927-8, *ibid.*, **54**, 292; 1928<sup>1</sup>, *Bacterial Immunity*, Stockholm; 1928<sup>2</sup>, *Zbl. Bakt.*, Abt. I, Orig., **108**, 294.  
 PFEIFFER, R., 1894, *Deuts. med. Wschr.*, **20**, 898; 1895<sup>1</sup>, *Z. Hyg. InfektKr.*, **19**, 75; 1895<sup>2</sup>, *ibid.*, **20**, 198; 1896, *Deuts. med. Wschr.*, **22**, 97, 119, 232.

PFEIFFER, R. & BESSAU, G., 1910, *Zbl. Bakt.*, Abt. I, Orig., **56**, 344.  
 PFEIFFER, R. & FRIEDBERGER, E., 1902<sup>1</sup>, *Berl. klin. Wschr.*, **39**, 4; 1902<sup>2</sup>, *ibid.*, **39**, 581; 1903, *Zbl. Bakt.*, Abt. I, Orig., **34**, 70; 1904, *ibid.*, Abt. I, Orig., **37**, 131; 1905, *Deuts. med. Wschr.*, **31**, 6, 1145; 1906, *Zbl. Bakt.*, Abt. I, Orig., **41**, 223.  
 PFEIFFER, R. & ISSAEFF, 1894<sup>1</sup>, *Deuts. med. Wschr.*, **20**, 305; 1894<sup>2</sup>, *Z. Hyg. InfektKr.*, **17**, 355.  
 PFEIFFER, R. & KOLLE, W., 1896<sup>1</sup>, *Zbl. Bakt.*, Abt. I, Orig., **20**, 129; 1896<sup>2</sup>, *Z. Hyg. InfektKr.*, **21**, 203.  
 PFEIFFER, R. & MARX, 1898, *Z. Hyg. InfektKr.*, **27**, 272.  
 POPPE, K., 1922, *Ergebn. Hyg. Bakt.*, **5**, 652.  
 RADZIEWSKY, A., 1901, *Z. Hyg. InfektKr.*, **37**, 1.  
 RODET, A., 1924, *C.R. Soc. Biol.*, Paris, **90**, 1262.  
 SAUERBECK, E., 1907, *Ergebn. allg. Path. path. Anat.*, **11**, 690.  
 SCHATTENFROH, A., 1897, *Arch. Hyg.*, Berl., **31**, 1; 1899, *ibid.*, **35**, 135.  
 SCHLEMMER, D., 1920, *Arb. Gesundh.Amt.*, Berl., **52**, 538.  
 SCHNEIDER, R., 1909, *Arch. Hyg.*, Berl., **70**, 40; 1911, *Zbl. Bakt.*, Abt. I, Ref., **48**, 271.  
 SCHÜTZE, A., 1904, *Zbl. Bakt.*, Abt. I, Ref., **35**, 274.  
 SHIBAYAMA, G. & TOYODA, H., 1906, *Zbl. Bakt.*, Abt. I, Orig., **40**, 566.  
 SINGER, E., 1925, *Z. ImmunForsch.*, **43**, 285.  
 SMITH, J. H. & BROOKS, R. St. J., 1912, *J. Hyg., Camb.*, **12**, 77.  
 SOBERNHEIM, G., 1913, *Handb. d. path. Mikroorg.*, hrsg. Kolle u. Wassermann 2nd ed., **3**, 583.  
 SORMANI, B. P., 1916, *Z. ImmunForsch.*, Tl. I, Orig., **24**, 336.  
 SPÄT, W., 1910, *Z. ImmunForsch.*, Tl. I, Orig., **7**, 712.  
 STEINHARDT, E., 1905, *J. Med. Res.*, **14**, 161.  
 THJØTTA, TH., 1920, *J. Immunol.*, **5**, 1.  
 TÖPFER, H. & JAFFÉ, J., 1906, *Z. Hyg. InfektKr.*, **52**, 393.  
 VAN DE VELDE, G., 1894, *Cellule*, **10**, 403.  
 WASSERMANN, A. & CITRON, J., 1905, *Deuts. med. Wschr.*, **31**, 1101.  
 WATANABE, S., 1919, *J. Immunol.*, **4**, 77.  
 WECHSBERG, F., 1902, *Z. Hyg. InfektKr.*, **39**, 171.  
 WEIL, E., 1905, *Arch. Hyg.*, Berl., **52**, 412, **54**, 149; 1909, *ibid.*, **70**, 173; 1911, *Wien. klin. Wschr.*, **24**, 229; 1921, *Z. ImmunForsch.*, Tl. I, Orig., **31**, 50.  
 WEIL, E. & FELIX, A., 1920, *Z. ImmunForsch.*, Tl. I, Orig., **29**, 24.  
 WILDE, M., 1902, *Arch. Hyg.*, Berl., **44**, 1.  
 WOLFF, A., 1903, *Berl. klin. Wschr.*, **40**, 414, 434, 456.  
 WRIGHT, A. E. & WINDSOR, F. N., 1902, *J. Hyg., Camb.*, **2**, 385.  
 ZINSSER, H., 1910, *J. Med. Res.*, **22**, 397.

## CHAPTER IX. HÆMOLYTIC ACTION.

BY R. MUIR (UNIVERSITY OF GLASGOW), WITH SECTIONS BY C. H. BROWNING (UNIVERSITY OF GLASGOW) AND S. P. BEDSON (LONDON HOSPITAL).

### Introduction.

IT had long been known that the serum of a normal animal may produce lysis of the red corpuscles of another species, and it was shown by Daremburg (1891) and by Buchner (1893) that this property was destroyed by heating at 55° C. The fundamental facts with regard to artificially prepared hæmolytic sera were, however, established by Bordet (1898, 1899). On injecting a guinea-pig with defibrinated blood of the rabbit he found that the serum of the former acquired markedly hæmolytic action against the rabbit's corpuscles, and that this action was destroyed on heating at 55° C. but was restored on the addition of fresh normal serum. He showed that the essential character of such a hæmolytic serum was that it contained a thermo-stable substance which combined with the corpuscles in a specific way, but which did not by itself produce lysis. He concluded that the specific substance in the immune serum acted as a sensitizer of the corpuscles (substance sensibilisatrice), so that the alexin of the normal serum, otherwise without action, entered into union with the corpuscles and produced solution of them. In this way he established the fact that the action of a hæmolytic serum proceeds on similar lines to that of a bacteriolytic serum. Bordet's work was followed by a series of papers by Ehrlich and Morgenroth (1899–1901), who made a detailed study of hæmolysis and brought forward additional facts.

In hæmolysis as in other immunity reactions there are two events to be considered, viz. the combination of the substances concerned—receptors, immune-body and complement—and the result of the combination, in this case lysis. The phenomena of combination will be afterwards considered, but at this stage it may be stated that the corpuscles may combine with many times the amount of immune-body necessary for lysis, and that the corpuscles thus sensitized may fix multiple doses of complement. The amount of complement fixed by a given quantity of red corpuscles along with the given amount of immune-body can be estimated according to the principles of a complement-fixation experiment. The estimation of the minimum hæmolytic dose of immune-body and complement accordingly gives no information as to the combining amounts possible—lysis is a phenomenon occurring at a stage, and often an early stage of the combining process.

**Nature of Lysis.**

As regards the nature of lysis it may be said to depend upon a partial solution of the envelopes of the corpuscles. The stromata are not destroyed, and can be seen under the microscope after lysis has occurred. Bordet (1899), however, showed that their physical properties are altered. As is well known, after corpuscles have been lysed with water the stromata contract when the fluid is made hypertonic and swell when the concentration is reduced below the isotonic point. When, however, the lysis has been effected by a lytic serum these phenomena are not seen ; the stromata remain practically unaltered in different concentrations of salt. Apparently then the lytic process has increased their permeability so that osmotic phenomena no longer occur. The effect of lytic action may be even more marked in the case of a bacteriolytic serum (Chapter VIII), almost complete solution of the bacteria being sometimes observed. Even in this case solution is, however, not quite complete, bacterial remains (Schatten) being left (Neufeld, 1908).

In view of the fact that no lysis occurs under the action of immune-body alone, the physical change in the corpuscles which leads to the escape of haemoglobin is usually attributed to the action of complement. In its lability it corresponds to many enzymes but its action is a quantitative one, depending on the amount of complement fixed. Scheller (1910), in accordance with Pfeiffer's opinion as to bacteriolysis, put forward the view that complement acts as a catalyser in relation to immune-body, the latter then exerting a ferment-like action ; and more recently Hill and Parker (1925) have come to a similar conclusion. In this connection, however, it may be noted that it was shown by Landsteiner and Jagić (1904) that silicic acid can take the place of a specific immune-body in acting along with normal serum and leading to lysis. The phenomenon has been minutely investigated and, although some minor differences have been found, the general result shows that complement is combined in the process and causes the lysis. The change produced in the erythrocytes by the silicic acid is closely analogous to that produced by the action of immune-body (see Landsteiner and Rock, 1912 ; Nathan, 1913 ; Browning and Mackie, 1914). This fact supports strongly the usual view, viz. that combined complement is the essential agent in lysis, and there appear to us to be no valid objections to it.

**Conditions Affecting Lysis.**

The amount and rapidity of lysis of a given amount of a suspension of red corpuscles depends on a variety of conditions—not only on the degree of sensitization and on the amount of complement, but also on the amount of fluid, salt-content, hydrogen-ion concentration, presence of protein, temperature, &c. The different factors concerned have been extensively studied but we can refer only to a few of the more important facts. It is also to be noted that when a fraction of the haemolytic dose of

immune-body or of complement is used, the amount of haemoglobin set free is not proportional. For example, if sensitized corpuscles in series are treated with progressively increasing fractions of the dose of complement, there is practically no lysis at first, then lysis occurs with comparative rapidity and this is followed by a tailing off of the amount of lysis until it is complete. This has been analysed graphically by Manwaring (1905), who was unable, however, to express the results as a mathematical equation. An accurate method of titrating lysis in proportion to the amount of complement has been given by Brooks (1919-20).

*The combination of immune-body and of complement* will be discussed more in detail later, but here some cardinal differences may be noted. As was first shown by Ehrlich and Morgenroth (1899<sup>1</sup>), immune-body combines readily with red corpuscles at 0° C., though with increasing rapidity up to incubation temperature. On the other hand, there is practically no union of complement at 0° C. This can be readily shown by exposing red corpuscles at 0° C. to the action of immune-body and sensitized corpuscles to the action of complement, and then centrifuging. In the former case immune-body is taken up by the corpuscles, whilst in the latter there is no union of complement. Again, union of immune-body with red corpuscles is of the reversible order and dissociation can be readily effected; this also depends on the temperature (p. 303). On the other hand, the union of complement is a firm one and it is doubtful whether it can be dissociated—that is, complement becomes fixed.

Another important point is that the amount of complement fixed by a given amount of corpuscles sensitized to a given degree, depends on the *concentration* of complement, not upon the amount present in the fluid; that is, in any given case more complement will be fixed when the fluid of suspension is smaller (Scheller, 1910). Thiele and Embleton (1914-15) confirmed this result so far as sensitization with a single dose of immune-body is concerned, but found that it is departed from with higher amounts of immune-body. On the other hand, the amount of immune-body entering into combination depends, within wide limits, on the amount present in the fluid, not on the concentration. Although the mode of union of immune-body differs from that of complement, it will be seen that, short of an imaginary point, we cannot speak of saturation with either. This may be put in another way by saying that in a given case when the union of immune-body or of complement has been completed, the addition of more immune-body or more complement leads to further combination.

These facts have an important bearing on the estimation of the minimum haemolytic dose of complement and of immune-body respectively. As is described elsewhere the haemolytic dose of immune-body is estimated in the presence of excess of complement (usually guinea-pig's serum), and that of complement in the presence of excess of immune-body. It must be noted that the minimum haemolytic doses of the two substances together do not cause complete lysis. Excess of immune-body accordingly, in

some way, aids the action of a given amount or rather concentration of complement, and excess of complement, in its turn, that of a given amount of immune-body. The degree to which this holds varies in the case of different immune sera and different complements. The quantitative relationships of immune-body and complement to lysis have been extensively studied (Morgenroth and Sachs, 1902; Noguchi, 1910; Hyde and Parsons, 1927), but it is not possible to give an accepted conclusion. As pointed out by Eagle and Brewer (1928-9), however, the phenomena may be theoretically explained in view of the modes of combination of complement and immune-body. Thus, on the one hand, an increased amount of immune-body leads to a more rapid and complete fixation of complement; and, on the other hand, with a given degree of sensitization a larger proportion of complement enters into combination when the complement is more concentrated. In either case there accordingly results the fixation of a greater amount of complement and therefore more lysis. One cannot, however, state at present that this is the whole explanation. The possibility of immune-body actually aiding lysis is considered later (p. 311).

The amount of lysis is influenced by the *salt concentration* and also by the nature of the salts present. Hæmolytic experiments are carried out in slightly hypertonic solution (0.85 per cent. of sodium chloride), and in such a medium lysis occurs most rapidly and with the smallest amount of complement. When, however, the salt concentration is progressively increased lysis becomes inhibited and ultimately prevented (Markl, 1902). This is due to prevention of the union of complement by the salt; the complement remains free in the saline solution and on dilution of the solution its action is restored. Up to a certain point the inhibition of a hypertonic solution may be compensated for by increased amount of immune-body, but beyond that point it is complete (Topley, 1915). Hypertonic solutions appear to prevent the adsorption of complement in general, and it was shown by Muir and Browning (1909) that whilst normal serum when filtered through a Berkefeld candle loses complement, the latter in hypertonic solution passes through and thereafter can be recovered by dilution. According to Eagle and Brewer (1929) complement fixation is prevented by those factors which suppress ionization of serum-proteins and lead to an increased aggregation state. In addition, various salts in isotonic solution inhibit lysis, in varying degree that is, in comparison with isotonic sodium chloride. This question was studied by Wright and MacCallum (1922), who found that variations depended both on the anions and the cations. For details their paper must be consulted, but it may be said that in general the salts with bivalent cations are more inhibitory than those with monovalent cations. In isotonic salt-free saccharose solution (9.5 per cent.), fixation and lytic action of complement do not occur, Sachs and Terruuchi, 1907). The lytic action can be restored in varying degree by different electrolytes, those with the lowest inhibitory power in saline being most effective in

this respect (Wright and MacCallum, 1922). On incubation at 37° C. complement in salt-free saccharose solution becomes destroyed after a time (Topley, 1915).

The *presence of protein* also exerts an inhibitory action on lysis. This is mainly due to the prevention of union of complement with the sensitized corpuscles (Muir and Browning, 1906). Lysis occurs less rapidly when the corpuscles are suspended in blood-serum and a much larger dose of complement is required than when saline is used as the medium. This fact may be taken advantage of in demonstrating slighter degrees of inhibitory action on lysis. For example, Bordet (1904) showed that an anti-immune-body prevented lysis when the corpuscles were in heated serum but not when they were in saline. The action of heated serum in interfering with lysis depends in part probably on its physical properties, but there is also evidence that complementoid may be concerned, that is complement which has had its lytic action destroyed by heating while its combining property is only impaired. The following result supports this view. When before heating the fresh serum, one removes its complement by means of absorbing agents, the retarding action of the treated serum when used as a medium of suspension is less marked and a smaller dose of complement suffices for lysis, than when the corpuscles are suspended in heated serum (Muir and Browning, 1906). The existence of complementoids has, however, in late years been called in question, but as yet no other explanation of the above has been given. It may be added that heated serum may interfere also with the union of immune-body with corpuscles (Ungermann and Kandiba, 1912), but as already stated the lytic action of complement is inhibited even when the corpuscles are fully sensitized. Heated serum may, on the other hand, increase the amount of lysis produced by a constant amount of immune-body and complement. This 'auxilytic' property has been described by Manwaring (1907), who found that its development might depend on the length of time during which the serum was heated. There is, however, the question of persisting portions of complement to be considered (Chapter X).

As regards *hydrogen-ion concentration* the optimum reaction for lysis is about the neutral point, according to Eagle and Brewer (1929) about pH 6·5 to 8. A change towards either the alkaline or acid side interferes with lysis. Up to a point the action can be restored by neutralization, but beyond this the complement is destroyed and the change is irreversible.

The results obtained from observations on lysis have been analysed by mathematical methods by Arrhenius (1907), McKendrick (1910-11) and Hill and Parker (1925) and equations have been given. It is not possible to give an account of this even in summary, nor do the results agree. McKendrick, for example, concludes from such a study that the action of complement is lytic, whilst immune-body after combination is a catalyst to complement. On the other hand, Hill and Parker find that

complement, during the time between its entrance into the cell and its combination, acts as a catalyst and allows the release of haemoglobin by the action of a ferment in the amboceptor. The mathematical side of the subject is considered also by Locke and Hirsch (1925) and by Eagle and Brewer (1929).

### Hæmolytic Receptors.

Immune-body combines with the stromata, and it is the stromata which act as antigens in the production of a hæmolytic serum (vide Chapter VI). Although it has been found recently that haemoglobin has some antigenic power, this never leads to the development of a hæmolytic serum, as has been shown by Hektoen and Schulhof (1922), Hektoen (1927) and by Yasui (1929) and others. The antigenic molecules of the corpuscles may be spoken of, in accordance with Ehrlich's terminology, as the receptors of the corpuscles. These receptors have considerable power of resistance to heat, as tested by their capacity to combine with immune-body *in vitro*. Muir and Ferguson (1906) showed in the case of ox's red corpuscles that the combining power becomes progressively impaired at 60° C., but a considerable amount still survives even after boiling at 100° C. [The receptors which have an affinity for Forssman's antibody are still more resistant to heating (p. 315).] Landsteiner and Prašek (1912) obtained a similar result and found further that horse corpuscles heated at 100° C. acted as antigens on injection into an animal. Landsteiner and Prašek showed also that a proportion of the receptors survived treatment with formaldehyde, with alcohol, and with ether. So also Sachs and Nathan (1913), by injecting boiled sheep's corpuscles, produced an immune-body, which was active also on the corpuscles of the goat (p. 305).

Hæmolytic immune-bodies may be produced by modified antigens. Landsteiner and van der Scheer (1925) have found that while alcoholic extracts of horse's corpuscles have little antigenic function in themselves, they have this property in considerable degree when mixed with foreign (e.g. pig's) serum. They have compared the antibodies developed against extract *plus* serum with those produced by injecting the corpuscles in the usual way, and find that certain differences exist. They find, for instance, that the action of the ordinary antihorse hæmolytic serum is not inhibited by the extracts but that of the antiserum obtained by extract *plus* serum is inhibited. They consider that the specific substances in the extracts are probably not proteins. When the corpuscles are suspended in isotonic glucose solution their receptors are more sensitive to the action of heat than when they are in saline; in the former medium a partial destruction occurs even at 55° C. (Meyerstein, 1923). It may be mentioned that corpuscles heated at about 55° C. and treated with multiple doses of immune-body constitute an active absorbing agent by which complement may be removed from a serum.

### The Union of Immune-Body.

The direct union of immune-body with the corresponding corpuscles can be readily demonstrated by the ordinary methods. Further details will presently be given with regard to the amount of immune-body which may enter into combination. A very important fact established by Ehrlich and Morgenroth in their first communication (1899<sup>1</sup>) is that the union of immune-body with red corpuscles takes place at 0° C.; whereas under the usual conditions complement does not unite with sensitized corpuscles at this temperature. There is thus afforded a useful method of separating immune-body and complement when they are present in a mixture. The temperature of the mixture is brought to 0° C., and there is then added a suitable amount of washed corpuscles at similar temperature. After an hour at 0° C. the immune-body will have united with the red corpuscles whilst the complement remains free in the fluid; this can readily be shown by the usual tests. It may be stated as a general law that immune-body of artificially developed haemolytic sera possesses the property of combining at 0° C. With normal haemolytic sera, however, the results vary; in some cases the immune-body unites with the corpuscles at 0° C., but in other cases a higher temperature is necessary for its union and then it may not be possible to separate immune-body from complement by the method described (p. 313).

The union of immune-body with corpuscles occurs with comparative rapidity at 37° C., being completed as a rule in a quarter of an hour. Owing to this high affinity of the immune-body for the red cells, its minimum haemolytic dose is, within fairly wide limits, practically independent of the volume of fluid in which the corpuscles are suspended (Scheller, 1910; Ungermann and Kandiba, 1912). At 0° C. the combination occurs more slowly, while above that temperature there is gradual acceleration of the process till about 37° C. Above the latter temperature the union becomes impaired (Cromwell, 1921; Meyerstein, 1923). In contrast to complement (p. 298), immune-body unites readily with red corpuscles in concentrated saline, though results vary with regard to details. According to Landsteiner and Welecki (1910), a certain amount of inhibition occurs, whereas v. Eisler (1909) and Angerer (1910) state that within wide limits combination is independent of the salt concentration.

An important fact in considering the relation of immune-body to red corpuscles is that in many instances the amount of the former necessary for lysis represents only a fraction of the total amount which will enter into combination. This can be readily shown by adding increasing amounts of immune-body to corpuscles in a series of test-tubes and after centrifuging, by finding the first tube in which a haemolytic dose of immune-body is free in the fluid. The number of haemolytic doses which enter into combination varies greatly in the case of different sera. Ehrlich and Morgenroth (1901<sup>1</sup>) found that with one serum (rabbit v. goat's corpuscles),

100 doses could be added before one dose remained free ; whereas with another serum (sheep v. dog's corpuscles), only a single hæmolytic dose was fixed. In the case of antiserum rabbit v. ox's corpuscles, the writer has found that usually from 6 to 8 hæmolytic doses give one dose free, but different samples of this serum vary considerably. It must be observed, however, that when we estimate by this method the amount of immune-body taken up by red corpuscles, we are using an arbitrary standard, as there is no sharp line of saturation. If, for example, when 10 hæmolytic doses are added 1 remains free, it will be found that with 9 hæmolytic doses there is nearly 1 dose free ; similarly 11 doses will give only a little more than 1 free dose, not 2 free doses. Within certain limits the amount of combined immune-body depends upon the amount of immune-body present. With any given quantity when equilibrium is established, there is a definite relation between the amount in combination and the amount which remains free. In this respect the phenomena correspond to those of mass action, and, as will be shown presently, the union is of the reversible order.

The quantity of immune-body which combines with red corpuscles in any instance may be estimated more satisfactorily by another method, namely, by adding immune-body to red corpuscles in great excess and then estimating the amount which remains uncombined in the fluid. The results thus obtained show in a striking way how much immune-body may enter into union and also how the amount absorbed by the corpuscles depends upon the amount present in the fluid of suspension. Cromwell (1922), for example, using a hæmolytic serum from the rabbit for sheep's corpuscles found that as much as 2,000 units (hæmolytic doses) could be taken up provided that the concentration of immune-body was sufficiently high. As an example—of 100 units added, 80 combined ; of 200, 160 combined ; of 1,000, 600 combined.

### Dissociation of Immune-Body.

The reversibility of the combination of immune-body with red corpuscles was demonstrated independently by Morgenroth (1903) and by Muir (1903). The latter employed the following method. To a suspension of red corpuscles, immune-body is added in great excess, time is allowed for combination and the corpuscles are then centrifuged and washed thoroughly with normal saline to remove the free immune-body—all the immune-body is then in combination. A similar quantity of suspension of untreated corpuscles is then added ; the mixture is centrifuged so as to bring all the corpuscles in contact, and is then placed in the incubator for an hour at 37° C. At the end of this time the addition of complement causes lysis of all the corpuscles. This, of course, means that a sufficient amount of immune-body has become dissociated from the sensitized corpuscles to sensitize the fresh corpuscles. If, on the other hand, the complement is added when the mixture of sensitized and fresh corpuscles

is first made, only the former undergo lysis, the complement being fixed by them and thus no longer available for the lysis of the untreated corpuscles.

Dissociation of immune-body from saturated corpuscles can also be shown by placing the corpuscles in the incubator for an hour after the free immune-body has been thoroughly removed by washing. It will then be found on centrifuging that a certain amount of immune-body is free in the fluid. The amount of lysis obtained in this way, however, is less than that occurring when the fresh corpuscles are actually in the presence of the saturated corpuscles ; this is apparently due to the fact that in the latter case the immune-body, as it dissociates, at once combines with the fresh corpuscles, and thus the process of dissociation is maintained. The amount and rate of dissociation occurring in any case depend upon the degree of firmness of the union of immune-body with red corpuscles. It thus varies much with different hæmolytic sera and even with different samples of the same type of serum. Thus with a rabbit's antiserum for ox's corpuscles the writer has found that with some batches 7 or 8 combined doses of immune-body will give off a hæmolytic dose under the conditions mentioned, but with other samples dissociation occurs in smaller degree.

The rate of dissociation depends on the temperature. The writer has found that scarcely any dissociation takes place at 0° C. and Philosophow (1909) obtained a like result. This fact is sometimes of service in determining whether immune-body is in the free or combined condition. For example, when red corpuscles laden with multiple doses of immune-body are lysed with a single dose of complement, we can determine whether the surplus immune-body is set free by the lysis or remains attached to the receptors of the red corpuscles. On adding fresh corpuscles in such a case at 0° C. it is found that practically no immune-body unites with them ; whereas at 37° C. a considerable amount of immune-body enters into combination. It would thus appear that when red corpuscles sensitized with multiple doses of immune-body are lysed, the surplus immune-body is not set free but remains in union with the receptors ; a certain amount, however, can be dissociated at suitable temperature. A detailed account of different conditions affecting dissociation of immune-body is given by Philosophow (1909) and by Morgenroth and Rosenthal (1912), but it may be mentioned that Philosophow showed that when the suspension of red corpuscles was in 2 per cent. sodium chloride the dissociation took place more rapidly than in 0·85 per cent. solution. Morgenroth and Rosenthal (1911) showed that the dissociability of an immune-body was increased when the antiserum had been kept for a long time, although this was not invariably accompanied by a marked increase in the hæmolytic dose. Similarly, deterioration of the immune-body brought about by heating at 65° C. for 30 minutes was accompanied by increased dissociability.

The fact that immune-body can be split off from sensitized corpuscles has been taken advantage of in methods for obtaining immune-body in

a concentrated form—as yet there is no question of a pure state being reached. Sheep's corpuscles especially have been used owing to the large number of units of immune-body with which they will combine. Kosakai (1918) found that dissociation of immune-body occurred more rapidly when the temperature was raised and also that it was greater when the corpuscles were in isotonic glucose solution than when in saline. Thus in glucose solution at 55° C. nearly all the immune-body was split off from the corpuscles. The glucose solution was treated with ether to remove lipins, immune-body not being extracted by this process; the glucose solution recovered by centrifuging was then dialysed to remove the glucose, &c. He thus obtained a substance with the properties of immune-body, the protein nature of which was doubtful. Huntoon and Etris (1921) and Meyerstein (1923) obtained a similar result as to dissociation in glucose solution. Immune-body can be dissociated from sensitized corpuscles by weak acids (v. Liebermann and v. Fenyvessy, 1909, 1911), but weak alkalis are more effective (Rondoni, 1910). The two first-mentioned observers were able to prepare a substance in a relatively pure condition which had the properties of immune-body and which gave only a feeble protein reaction.

Coulter (1921) has found that in a salt-free saccharose medium the proportion of free and combined immune-body reaches a similar equilibrium whether the immune-body is originally in the free or in the combined state. The amount combined is essentially related to the isoelectric point of the immune-body, not of the red corpuscles, and reaches its maximum at pH 5·3, where it is nearly 100 per cent. The addition of alkali or acid increases the amount in the free state, alkali acting more rapidly in this respect. At a pH of about 9, over 90 per cent. of immune-body is free. The presence of sodium chloride increases the amount combined at all points except about the isoelectric point.

Locke and Hirsch (1925) prepared the stromata of sheep's corpuscles saturated with immune-body and after removing the haemoglobin obtained the stromata as a whitish powder. After treatment with ether the stromata were washed with saline and were then treated with weak sulphuric acid which extracts the immune-body. The latter was then precipitated by neutralization. The final product, which was soluble in saline, contained immune-body in an extremely concentrated state, but still gave a positive protein reaction.

Dissociation of immune-body occurs also within the animal body when sensitized corpuscles are injected. The antigenic properties of the corpuscles are diminished, but a certain amount of immune-body may be developed in response to them. This, of course, might be due to the fact that it is difficult to ensure complete saturation, and some of the receptors might be free from immune-body and act as antigen. When, however, an animal (e.g. the rabbit) is injected with its own corpuscles sensitized with immune-body, the lysis which results is not confined to the sensitized corpuscles but also occurs in the blood generally (Muir and McNee, 1912-13).

This can only be explained by dissociation of immune-body from the corpuscles injected. It may be mentioned further that Altmann (1912) showed that when corpuscles laden with immune-body are injected into an animal of alien species the immune-body becomes dissociated and then acts as antigen.

### Specificity of Immune-Body.

The nature of the specificity of immune-bodies is well illustrated by certain facts which can be readily demonstrated in the case of hæmolytic sera. These facts depend upon the circumstance that in any organic material or organized structure used as antigen all the molecules or aggregates which have antigenic function are not alike in structure. Many of them may be peculiar to the organism from which the antigen is derived, but others may form part of other organisms. This principle was, we believe, first put forward by Durham (1900-1) in explanation of the fact that an agglutinating serum for a particular bacillus may cause agglutination of allied bacilli, though in a feebler degree. The existence of what may be called partial hæmolytic immune-bodies was first demonstrated by Ehrlich and Morgenroth (1901<sup>2</sup>). They found that an immune-body obtained from the rabbit for ox's corpuscles acted also on goat's corpuscles, though the action on the latter was weaker. Similarly, an immune-body for goat's corpuscles acted also on ox's corpuscles—the hæmolytic dose for the latter being again higher. On treating the anti-ox serum with goat's corpuscles, the hæmolytic action for these was removed, though the serum still possessed considerable hæmolytic action for ox's corpuscles. A corresponding result was obtained when an antigoat serum was absorbed with ox's corpuscles, the action on the latter being again removed. Each serum thus contained a component which was absorbed by goat's and ox's corpuscles alike and each a part absorbable only by the corpuscles used as antigen. Other examples of the same principle have been found in the case of other allied species—sheep and goat, ox and sheep, monkey and man, &c. Ehrlich and Morgenroth (1900<sup>1</sup>) found that even an isolysin for goat's corpuscles (p. 307) produced also solution of sheep's corpuscles. The term specificity is thus to be used in relation to the structure of the constituents of an antigen, we may say to their combining affinities ; and while in an antigen there may be one chief component peculiar to the species there are also other components common to allied species.

When immune-bodies for the same corpuscles obtained from different species of animals are compared, the following facts are brought out. The immune-body from species A may prevent the combination of immune-body from species B (Muir, 1911-12). The two immune-bodies in such a case apparently unite with the same receptors and each excludes the other ; this cannot, however, be said to hold universally. In other respects the immune-bodies have different properties. For example, the dose of a given complement varies in the two cases ; that is, the action of

complement depends upon the immune-body by which it is brought into relation with the corpuscles. As an extreme case the complement may fail to produce lysis with one immune-body while it does so with another. Again, immune-bodies from different species vary in respect of the number of haemolytic doses which unite with the same corpuscles and the firmness of union ; they vary also as regards the amount of complement fixation which they lead to. Another important fact bearing on this subject is that an anti-immune-body (p. 308) is effective only against the immune-bodies of the animal against whose serum it has been developed. It is doubtful whether this represents an antibody reaction against the immune-body as such or merely a specific antiprotein reaction. The general outcome of work on this subject has thus been to show that immune-bodies possess specificity of two kinds—(a) they are specific in relation to the antigens used for their production and (b) they are specific in respect of the animal from which they have been derived.

#### **Qualitative Variation in Immune-Body.**

It has been established that a haemolytic immune-body has not a uniform character at different stages of immunization but undergoes certain qualitative changes. As has been stated above red corpuscles can combine with multiple doses of the homologous immune-body and thereafter there is a corresponding, though not strictly proportional, fixation of complement ; in this way many times the amount of complement necessary for lysis can be fixed. We may thus speak of the fixation of complement through the medium of immune-body. Browning and Wilson (1911) showed that at an early stage of development of immune-body in the rabbit for ox's corpuscles, the immune-body was deficient in the power of fixing complement ; there was only a slight increase in the amount of complement fixed under the influence of multiple doses of immune-body as compared with the amount fixed by one dose. Later in immunization the fixing power increased. On the other hand, if the amount of immune-body in the blood was falling after the injection of red corpuscles had been stopped, the complement-fixing power of immune-body remained high even when the haemolytic dose had fallen to a considerable extent. Thiele and Embleton (1914) described various qualitative changes in the development of immune-body. They found that the haemolytic power which at first appears is due to a complement-like thermo-labile substance which does not unite at 0° C. ; a little later, union of immune-body, still thermo-labile, occurs at this temperature. Thereafter the lysin comes to have the dual constitution of immune-body and complement, but when this is first established the immune-body fixed at 0° C. is still thermo-labile ; later it acquires the ordinary thermo-stable character.

Cromwell (1922), in studying the relative proportions of immune-body absorbed and left free in the fluid when different amounts of immune-body were used, obtained results which pointed to a deterioration in the

sensitizing power of immune-body in the later period of immunization. Fall in the titre or hæmolytic power of the serum was found to be associated with a fall in the number of hæmolytic doses of immune-body which combine with the corpuscles. This is apparently due to the absorption of modified immune-body which keeps active immune-body out of combination, but which in itself does not act as a sensitizer. He found that red corpuscles treated with a serum giving low absorption of active immune-body took up less additional immune-body than corpuscles which had absorbed a larger number of hæmolytic doses from a serum with higher absorptive property. The serum that gives the higher absorption of immune-body has the smaller proportion of inhibiting substance and the latter may be regarded as modified immune-body.

Müller (1908) studied the combination of the molecules of an immune-body and found that differences in their avidity for the corresponding corpuscles existed. The avidity was estimated by finding in a mixture of immune-body and corpuscles the proportions of free and combined immune-body present after time is allowed for complete union. He used the term 'absorption coefficient' to indicate in such a case the ratio of combined immune-body to the whole immune-body present. Estimating first the absorption coefficient in a particular case, he found that the fluid separated from treated corpuscles gave a lower absorption coefficient than the original solution of immune-body. (On the other hand, when a solution of immune-body is diluted its absorption coefficient is higher; that is, a larger proportion of immune-body is absorbed by red corpuscles.) This appears to be due to the fact that the immune-body is not homogeneous but is composed of molecules with varying affinity for red corpuscles, those with greatest affinity being absorbed first. He found that in the process of immunization there is a gradual increase of the combining power of immune-body, as regards velocity of reaction and amount entering into union, the antibody molecules formed at an early period reacting less strongly than those at a later. Hence at any given stage the molecules vary in their affinity for red corpuscles. The results of Morgenroth and Rosenthal (1911), already referred to afford additional evidence with regard to qualitative changes in immune-body (p. 303).

### Isolysins.

The term isolysin was introduced by Ehrlich and Morgenroth (1900<sup>1</sup>) to indicate a hæmolytic immune-body obtained by injecting an animal with the corpuscles of other animals of the same species—in contrast to the ordinary hæmolysin or heterolysin developed against the corpuscles of another species. These observers injected each of three goats with the mixed corpuscles of three other goats and found that in every case an isolysin developed which dissolved the corpuscles used in immunization. Not only so, but each acted on the corpuscles of other goats than those whose corpuscles had been used for injection; this was found to be the case in a considerable number of instances though not in all. The three

isolysins were also found to lyse sheep's corpuscles (a fourth isolysin which they obtained, however, did not act on sheep's corpuscles). They showed that the isolysins varied in the manner and degree in which they affected the corpuscles of various goats and thus were to be regarded as differing in constitution. In no case had an isolysin any effect on the corpuscles of the animal from which it was obtained ; that is, an isolysin never acted as an *autolysin*. Isolysins have been developed in other animals, for example in dogs, by Ottenberg, Kaliski and Friedman (1913) and in chickens by Hada and Rosenthal (1913).

Striking results of similar nature were obtained by Todd and White (1910, 1911-12) in the case of cattle treated in the process of immunization against cattle plague, the animals in question having received injections in large amount of the blood of other cattle. The sera of cattle thus treated were found to have lytic action on the corpuscles of other cattle when guinea-pig's serum was used as complement, but in no instance on the corpuscles of the animal from which the serum was obtained ; each serum varied in the degree of its haemolytic property on the corpuscles of individual animals. Lytic action against goat's and sheep's corpuscles also was found to be developed. On making a polyvalent serum by mixing the sera of a large number of immune animals and then absorbing the serum by corpuscles of an individual animal, it was found that the lytic property for these corpuscles was removed, while the serum still contained lysin for the corpuscles of other animals, unless the latter were closely related genetically to the animal whose corpuscles had been used for developing the serum. In this way it was shown that the corpuscles of an animal possess a certain individuality of their own—that is, a certain degree of difference from those of other animals. It is this slight degree of difference or foreignness that confers on them the antigenic function. It may be added that Todd (1930) has recently published quite analogous results obtained by agglutination methods in the case of fowls. Here also it was found possible to differentiate the red corpuscles of any individual fowl by means of serum reactions, except in cases where there was close blood-relationship.

In no instance has an autolysin been experimentally produced and the theoretical considerations underlying this fact were discussed by Ehrlich and Morgenroth. These need not be entered into, but it is evident that the absence of autolysin-production represents a phenomenon of adaptation, without which antibodies would be developed against an animal's own cells.

#### The Question of Anti-Immune-Bodies.

The serum of a normal animal contains natural immune-bodies of various kinds, and these have antigenic function. Bordet (1904) showed that when the serum of an animal is injected into another of different species there are developed in the latter anti-immune-bodies to the immune-bodies of the former, the presence of which can be shown by

haemolytic experiments. The anti-immune-bodies thus developed are effective not only against the natural immune-bodies of the serum injected but also against immune-bodies which may be artificially formed in the species of animal supplying the serum. The antibodies act by preventing the union of complement with the sensitized corpuscles, not by interfering with the union of immune-body with the corpuscles. Ehrlich and Sachs (1905) confirmed Bordet's results and regarded them as supporting strongly their view as to the amoceptor constitution of immune-bodies. The action of anti-immune-body was shown by Bordet as follows. Red corpuscles are sensitized by an immune-body and are washed free of serum; they are then treated with the corresponding antiserum containing anti-immune-body and again washed. When they are suspended in heated serum (e.g. guinea-pig's) and complement is added lysis does not occur. The heated serum is used as a medium of suspension, since lysis occurs less readily and requires a larger amount of complement than when the corpuscles are suspended in saline. Even, however, when they are in saline the action of the anti-immune-body is shown (*a*) by lysis occurring more slowly and (*b*) by the anti-immune-body interfering with the fixation of complement by the sensitized corpuscles (Muir and Browning, 1906). Apparently the union of anti-immune-body with the sensitized corpuscles is comparatively loose and dissociation of it may occur. While there is no doubt that anti-immune-body prevents the union of complement, a fact which has been confirmed by Browning and Sachs (1906), the writer failed to find evidence that the converse holds good—that is, that the union of complement prevents the union of anti-immune-body with sensitized receptors.

An interesting effect opposite in nature to the inhibition of lysis was observed by Friedberger and Moreschi (1908). They found that when red corpuscles sensitized with immune-body and washed free from the antiserum are treated with an antiserum to the serum of the species of animal supplying the immune-body, haemolysis is favoured and occurs with a smaller dose of complement. Apparently the combination of serum *plus* antiserum on the corpuscles leads to the fixation of complement and intensifies its action. It will be noted that both the antagonistic action of anti-immune-body described above and this favouring effect are brought about by antibodies to the serum of the animal species supplying the immune-body tested. In some instances one effect results, whilst in others the opposite property appears, and without further knowledge of the constitution of an immune-body it is not possible to give an explanation of the discrepancy. It is possible that both effects are due to a protein-antibody complex on the surface of the erythrocytes, this complex in some instances leading to the union of additional complement with intensification of lysis, in others interfering with the union of complement. Theoretically, however, one might expect that such a complex would simply fix complement, but the striking fact is that in the anti-immune-body reaction the combination of complement is prevented. The relation

of the two effects is still doubtful (see Sachs, 1929). Altmann (1912) found that immunizing an animal with the sensitized and washed corpuscles of an animal of another species leads to the formation of a precipitin for the serum of the animal supplying the immune-body, and thus to the Friedberger-Moreschi phenomenon. What has been added to the corpuscles in the process of sensitization thus acts as an antigen in the body of the animal used for injection ; but it is not possible to say whether this is the immune-body itself or associated protein.

### The Nature and Mode of Action of Immune-Body.

In their first communication on hæmolsins, Ehrlich and Morgenroth (1899<sup>1</sup>) put forward the view that immune-body possesses two combining groups ; by one of these it combines in a specific manner with the receptors of the red corpuscles, &c. ; while by the other, called ' complementophile ', it fixes complement. The immune-body is thus supposed to act as an intermediary between the cell receptor and complement, and Ehrlich accordingly gave it the name of *amboceptor*, a term which has come into fairly general use. Ehrlich also held that the union of complement with immune-body actually occurs at higher temperatures, that the union is of loose character and that dissociation occurs at lower temperatures ; on the other hand, the union becomes firm when once the immune-body has combined with receptors. Bordet, however, has consistently maintained that the immune-body leads to some physical change in the bacterium or corpuscle and sensitizes it, so that complement enters into combination. He holds also that complement in its combining affinities does not show any specific character such as is seen in the union of antigen and antibody. The question has been the subject of long controversy and cannot be discussed in detail ; but some facts may be mentioned in connection with it.

In support of his view Ehrlich put forward the fact that complement acts best in association with immune-body when they are both from the same species of animal. This, however, although suggestive, does not appear conclusive, as it seems that it might be the case according to Bordet's view ; seeing that the two substances act somehow in co-operation, it is likely that this would be most effective when they are from the same animal. Again, an ' anti-immune-body ' unites with immune-body in a specific way and acts by preventing the union of complement. This fact appeared to be of considerable weight as it pointed to anti-immune-body and complement combining with the same group of the amboceptor ; but another explanation is possible (p. 309). On the other hand, many complements of different kinds are brought into union with the corpuscles by the same immune-body, and further it has not yet been shown that the union of complement prevents the union of anti-immune-body. The following experiment of Ehrlich and Sachs (1902) has been frequently quoted as supporting the view that complement combines directly with

immune-body. Guinea-pig's corpuscles treated with natural immune-body from the ox (heated ox's serum) and then washed do not undergo lysis on the addition of horse's serum as complement, but do so when the immune-body and complement are added together. It was supposed, therefore, that the natural immune-body for the guinea-pig's corpuscles has little affinity for them until it has combined with complement. The problem cannot be discussed in detail here, but it may be said that analysis of the phenomena by Bordet and Gay (1906) and Yoshinare (1922) has shown that there is no evidence which implies a direct union of immune-body and complement.

Against Ehrlich's view is the fact that direct union of immune-body and complement has not been demonstrated. The dissociation supposed by Ehrlich to occur at lower temperatures must be an extremely rapid one as the immune-body in its combination at 0° C. never carries complement with it; and Arrhenius (1907) considered on grounds of physical chemistry that such dissociation was very improbable. Again, when immune-body and complement are present together in hypertonic salt solution, the former unites readily with the corpuscles, though some interference may occur, but the union of complement is prevented even at 37° C.; dissociation must accordingly be brought about by the salt solution. Muir and Browning (1909) showed that when fresh serum is filtered through a Berkefeld filter the complement in the early period of filtration is retained by the filter; diluted immune-body, on the other hand, passes through practically unchanged. When the complement and immune-body are mixed and filtered at warm temperature, the same result follows—the complement is retained while the immune-body passes through. This is against Ehrlich's view, seeing that if there was actual combination complement absorbed by the filter would keep back a certain amount of immune-body. Muir (1903) showed that when large amounts of complement had been fixed to the receptors of red corpuscles through the medium of immune-body, the immune-body could be dissociated while the complement remained fixed. Apparently the latter had entered into direct union with the receptors of the corpuscles. In view of all the facts it appears that the direct union of complement and immune-body has not been proved. The general results of recent work go to show that the fixation of complement in any antigen-antibody reaction—whether by sensitized erythrocytes, sensitized bacteria or serum precipitate—is of similar nature and probably depends upon changes in molecular aggregation. Whether it is of the same nature in intensified form as the direct adsorption of complement by organic or inorganic particles cannot be definitely stated. The facts established, however, with regard to precipitating sera clearly show to how great a degree complement fixation depends upon physical conditions.

Although immune-body produces no lysis by itself there is still the possibility that it may in some way aid the process, in addition to leading to the union of complement. Some facts bearing on this point may be

given. When two immune-bodies from different animals for the same corpuscles are used, there may be considerable differences in the dose of a given complement necessary for lysis. To make a comparison it must be shown that the complement in each case is fixed, and this can readily be done in many cases. A certain amount of sheep's complement produces lysis of ox's corpuscles sensitized with goat's immune-body, while the same amount causes only slight lysis with rabbit's immune-body ; but complement is fixed in both instances. The difference in the result might be due to the two immune-bodies uniting with different receptors of the corpuscles ; but for this there is no evidence. v. Poggendorf (1909) found that the goat's immune-body unites with the same receptors as the rabbit's immune-body, each immune-body keeping out the other ; and Muir (1911-12) obtained the same result. Again, horse's complement does not lyse ox's corpuscles sensitized with rabbit's immune-body but does so with cat's immune-body ; the complement is fixed in both cases and the two immune-bodies can be shown to unite with the same receptors of the red corpuscles. It is therefore not immaterial by what immune-body the complement is brought into union with the corpuscles. With the same complement acting on the sensitized corpuscles the lytic result varies according to the immune-body used to sensitize them. These facts suggest that immune-body plays some auxiliary part in the lytic process and does not merely lead to linking of the complement to the corpuscles. The results of Cromwell (1922) point in the same direction.

As regards Bordet's view that the immune-body produces some physical change in the red corpuscles, it is to be noted that Landsteiner and Jagić (1904) found that treatment with colloidal silicic acid rendered red corpuscles sensitive to the action of complement (see Browning and Mackie, 1914). Mudd and Mudd (1926) have shown that the process of sensitization of the erythrocytes is accompanied by physical change. They studied the behaviour of unsensitized and sensitized corpuscles at the interface between two immiscible liquids, oil and saline, and found that the former possess only a slight margin of stability and pass readily into the oil, whereas the sensitized corpuscles have much greater stability and pass into the oil only with considerable mechanical aid. Corresponding effect is described in the process of sensitizing bacteria to phagocytic action (Chapter XI). Reiner and Fischer (1929) have supposed that the immune-body acts on the red cells as a dehydrating agent, since corpuscles which have been treated with tannic acid and washed undergo lysis on the addition of complement, but are not affected by heat-inactivated serum. However, agglutination of the red cells which is caused by tannic acid, is not invariably brought about by hæmolytic immune-body ; indeed, the latter may have no other observable effect than to sensitize the corpuscles to the action of complement.

**Natural Immune-Bodies.**

It has long been known that the normal sera of animals may possess lytic action on the erythrocytes of other species, and, as has been stated above, this property like bactericidal action is destroyed at 55° C. Ehrlich and Morgenroth (1899<sup>2</sup>) were the first to show that in this normal lysis two substances, a natural immune-body and complement, are concerned. In some instances the action of the two substances can be readily demonstrated by applying the principles already described. For example, normal guinea-pig's serum possesses some lytic action on ox's corpuscles, and if the serum is treated with the corpuscles at 0° C. the natural immune-body is fixed to the corpuscles, while the complement remains free in the serum. The serum is then without lytic action, while the corpuscles have undergone a certain degree of sensitization. The same holds in the case of lytic action of dog's serum on guinea-pig's corpuscles, and the corpuscles sensitized at 0° C. undergo lysis even in guinea-pig's serum. But in many instances separation of the two constituents is attended with much difficulty. This is due to the fact that there may be little or no combination of the natural immune-body with corpuscles at 0° C., union occurring only at higher temperatures. And again, when inactivation by heat is applied it is found that in some instances the natural immune-body is almost as thermo-labile as is the complement. For example, the natural immune-body of dog's serum for guinea-pig's corpuscles is largely destroyed at 55° C., while the complement is destroyed at a still lower temperature (Sachs, 1902). Inactivation without destroying the natural immune-body may be effected on varying the temperature, but even this method may fail. The demonstration of immune-body and complement in normal ox's serum for guinea-pig's corpuscles cannot be effected by the two ordinary methods. In this instance Yoshinare (1922) found that when the serum was passed through a Berkefeld filter the complement was retained whilst immune-body passed through. The facts established show that the lytic action of a normal serum is the prototype of that seen in the case of artificially prepared hæmolytic sera and that as a rule an immune-body and complement are concerned; though for the reasons mentioned their separation may be a matter of considerable difficulty.

In certain instances investigated, the dual constitution of the hæmolysins of cold-blooded animals has not been established and some observers consider that toxins rather than complements are concerned (Friedberger and Seelig, 1908). The splitting of the lytic agent into fractions like those of complement (Chapter X) has, however, been effected in the case of eel's serum by Liefmann and Andreew (1911), and by Landsteiner and Rock (1912) in the case of frog's serum. The latter observers showed also that the frog's serum reactivated mammalian immune-bodies and that its lytic properties were removed by complement-fixing agents. The subject is one of which, however, further investigation is needed.

**The Combination and Lytic Action of Complement.**

The properties of complement are discussed in detail in Chapter X, but some facts brought out by hæmolytic experiments may be given here. We may first refer to variations in the lytic dose of complement, that is, in the amount of a given fresh serum necessary for lysis. In this connection there are two main factors, viz. (a) the combining relationships of complement and (b) its relative toxicity for the corpuscles in question. Any lytic action of complement implies combination and the latter is of firm character, there being no satisfactory evidence that complement is recoverable after it has combined. When combination of complement has occurred (as shown by the ordinary type of fixation experiments) and the hæmolytic dose is high, this is often due to the fact that the corpuscles are little sensitive to the action of the complement. In many instances the corpuscles of an animal are relatively insensitive to the complement of the same animal, no matter by what immune-body the union of complement has taken place. Muir and Browning (1904) showed that this was the case with the action of rabbit's and guinea-pig's complement towards the corresponding corpuscles when sensitized. The most striking example, however, is seen in the relation of ox's complement to ox's corpuscles, no lysis occurring when four different immune-bodies were tested (Muir, 1911-12). Even when an isolysin is used no lysis occurs, although the isolysin along with a small amount of guinea-pig's complement causes complete lysis. Relative toxicity of the complement towards the corpuscles is thus an important factor.

Variations in the hæmolytic action of different fresh sera may depend on variations in what we may call the amount of complement molecules present in the serum. Muir and Browning (1904) found this to be the case on comparing guinea-pig's and rabbit's sera, the hæmolytic dose of the latter being much the higher. If complement is in a concentrated form in guinea-pig's serum (the hæmolytic dose of which is small) a small amount of guinea-pig's serum should prevent the union of a corresponding amount of rabbit's complement as tested by hæmolytic doses; and conversely a relatively large amount of rabbit's serum should be necessary to keep out a small amount of guinea-pig's complement. This was found by experiment to be actually the case.

Complements vary as regards the amount in which they are taken up or fixed by means of multiple doses of immune-body, and variations in this respect are seen also when a given complement is used along with different immune-bodies. It can often be shown that the amount of complement fixed increases when multiple doses of immune-body are used. To corpuscles sensitized with multiple doses of immune-body in a series of tubes, increasing amounts of complement are added, and then after allowing time for combination sensitized corpuscles are added to each tube. The non-occurrence of lysis, of course, shows that the complement has been fixed. The relative quantity of complement which

may be fixed in this way varies greatly in different instances. For example, ox corpuscles sensitized with 10 doses of immune-body from the rabbit will fix approximately 10 doses of guinea-pig's complement ; whereas of cat's complement little more than that necessary for lysis is fixed, and this is the case even when cat's immune-body is used. The deviability or degree of fixation of complement thus varies much in different instances.

Sometimes a given complement fails to produce lysis of sensitized corpuscles. This failure may be due to two causes : (a) the complement may fail to combine with the sensitized corpuscles and (b) union may actually occur but no lysis follows. As an example of the former—failure of combination—may be mentioned guinea-pig's complement along with ox's corpuscles sensitized by immune-body from the duck ; the complement remains free in the fluid and no lysis occurs (Muir, 1911-12). A striking example of combination of complement without lysis is seen in the case of horse's complement along with ox's corpuscles sensitized with immune-body from the rabbit (Browning, 1906 ; Bordet and Gay, 1906). Another instance is afforded by the behaviour of ox's complement towards ox's corpuscles ; in this case complement may be taken up through the medium of various immune-bodies without any lysis resulting. Accordingly when a complement along with a given immune-body does not produce lysis, it must not be assumed that the complement does not 'suit the immune-body', that is, is not brought into combination by means of it.

### Forssman's Heterophile Antibody.

By C. H. BROWNING.

Forssman (1911) demonstrated the remarkable fact that when watery suspensions of guinea-pig's organs were injected, e.g. intraperitoneally, into rabbits a powerful lytic immune-body for sheep's red corpuscles developed—'Forssman's antibody' (see Taniguchi, 1921<sup>1&2</sup>). This antibody has also been designated 'heterophile' (Friedemann, 1917), or 'heterogenetic' (Friedberger and Schiff, 1913), since it reacts with an antigen (Forssman's antigen) derived from a species of animal which is not closely related to that which stimulated its production. It is to be noted that the response of individual animals to this antigen varies greatly (Mackie, 1925) ; but a potent antiserum may result from a single injection. The heterophile immune-body is absorbed when the antiserum is treated either with suspensions of tissues which contain the corresponding antigen or with sheep's red corpuscles. The antiserum contains very little lytic immune-body for guinea-pig's blood corpuscles. There is strong evidence, therefore, that the development of Forssman's antibody represents a real immunity response, although the view has been held that it is due merely to increase of normal antibody resulting from a non-specific stimulus (Friedberger and Schiff, 1913).

In addition to its action as hæmolytic immune-body for sheep and goat corpuscles, and also for the red cells of the hen (Kritschewsky, 1923) and some other species (see Friede and Grünbaum, 1925), the antiserum agglutinates sheep's corpuscles, provided that these have been kept for several days after withdrawal of the blood (Trou-Hia-Hsü, 1922). Further, it is of very great importance that Forssman's antibody reacts *in vitro* with the lipoids extracted by alcohol from tissues which contain the antigen (Iwai, 1917; Georgi, 1919; Taniguchi, 1921<sup>1 & 2</sup>). The reacting substances occur in the constituents of the alcoholic extract which are soluble in ether but insoluble in acetone, i.e. the lecithin fraction, and they withstand boiling in alcohol for several hours; but their chemical nature is not yet known (see Landsteiner and Levene, 1926-7). The main evidences of reaction are as follows: (1) when the antiserum is mixed with a saline emulsion of alcoholic extract of tissues containing Forssman's antigen specific combination occurs, so that the sensitization of subsequently introduced sheep's red cells is interfered with; (2) mixtures of the antiserum with Forssman's lipoids show precipitation and also have the property of fixing complement. As in the case of the Wassermann reaction, these phenomena are intensified by the addition of cholesterol to the solution of lipoids and also by increasing the turbidity of the emulsion, this being effected by slow admixture of the alcoholic solution with saline. Another property of sera containing Forssman's antibody is that on injection into normal guinea-pigs they cause symptoms like those of anaphylaxis—primary or heterophile toxicity (see Taniguchi, 1922; also Chapter XV).

Investigations on the distribution of Forssman's antigen in various animal species (see Forssman, 1928) have shown that, in addition to being present in guinea-pig's tissues, it occurs in varying proportions in the tissues of many warm- and cold-blooded animals, e.g. cat, dog, horse, mouse, hen, tortoise, &c., hence these are called animals of 'guinea-pig type'. It has been demonstrated also in urine, e.g. of the horse, in transplantable carcinoma tissue of the mouse as shown by Morgenroth and Bieling (1915) and in hen egg yolk (Iwai, 1917); further, the antigen is present in certain bacteria, e.g. some strains of Shiga's dysentery bacillus (Iijima, 1923; Yasui, 1929<sup>2</sup>), and in certain strains of the bacillus of rabbit septicæmia although absent from most (Powell, 1926). On the other hand, the tissues of the rabbit, ox, pig, goose, toad, &c.—animals of 'rabbit type'—lack this antigen; it is absent also from human tissues, but red corpuscles containing the A-receptor are a possible exception. Boiled suspensions of the active tissues are also effective antigens (Doerr and Pick, 1913). It is striking that while Forssman's antigen is present in the organs of the mouse, it does not occur in those of the closely related rat. Further, in a number of other animals presumptive evidence of the presence of the antigen has been afforded by the fact that their tissues fix Forssman's antibody *in vitro*; human red corpuscles containing the A-receptor have this property (Schiff and Adelsberger, 1924; Amzel,

Halber and Hirsfeld, 1925 ; Landsteiner and van der Scheer, 1925<sup>2</sup>). Only the animals of rabbit type are capable of developing heterophile antibody. Accordingly, the presence of Forssman's antigen in the tissues on the one hand and, on the other, the capacity to develop heterophile antibody—with which capacity is commonly associated the presence in the serum of natural antibody of this type—appear to be mutually exclusive characters (see Rubinstein, 1927).

The capacity of a tissue to act as Forssman's antigen *in vivo* corresponds in general with the capacity of watery suspensions or of the extracted lipoids to react with the antibody *in vitro*, although the binding power of different tissue suspensions may vary greatly. Thus Forssman (1911) stated that guinea-pig's liver failed to fix heterophile antibody, but Orudschiew (1913) showed that binding power was present although weak. According to Bail and Margulies (1913), in such cases boiled suspensions combine with the antibody more intensely than unboiled. The latter workers have shown also that treatment of fresh suspensions of guinea-pig's organs with unheated or heated normal sera of animals of rabbit type generally deprives them of the power to fix heterophile antibody, whereas sera of animals of guinea-pig type are without such action. Again, as judged by their capacity to stimulate the formation of Forssman's antibody in rabbits, some organs of an animal may contain more of the antigen than others ; while from certain tissue elements it may appear to be absent altogether. For example, the red corpuscles of the sheep or goat lead to the development of immune-body, a moiety of which possesses heterophile characters (see p. 318), but the organs of these animals are devoid of this property. Similarly, guinea-pig's red blood corpuscles do not stimulate the production of Forssman's antibody, whereas practically all the other tissues including connective tissue, but especially kidney and lung, are effective, and even serum has some action. Accordingly it has been concluded that when Forssman's antigen is present in the tissues it is absent from the red corpuscles of that species and vice versa ; but this does not appear to hold universally (see Friede and Grünbaum, 1925). Witebsky (1927) has observed, however, that the lipid constituent of Forssman's antigen is actually present in alcoholic extracts of sheep's kidney and guinea-pig's red cells, as shown by the complement-fixation reaction along with heterophile antibody (antiserum from rabbits injected with a mixture of alcoholic extract of horse kidney *plus* pig's serum, see p. 318). According to this investigator, guinea-pig's corpuscles contain, in addition, a specific lipid which when injected into rabbits, following the procedure of Landsteiner and Simms, leads to the production of hæmolytic immune-body for the corresponding red cells. In accordance with the principle of 'competition of antigens' (see Heimann, 1927) the latter lipid therefore appears to inhibit antibody production in response to the Forssman antigen which is also present.

A striking difference between Forssman's antibody and immune-body obtained by injecting sheep's corpuscles into the rabbit is that the former

does not lead to haemolysis of ox's red cells. According to Friedberger and Schiff (1913), however, ox's corpuscles also do not combine with the immune-body got by injecting sheep's corpuscles into guinea-pigs. The heterophile antibody also is fixed in much larger quantity by sheep's corpuscles and dissociates again less readily than the homologous immune-body v. sheep corpuscles derived from guinea-pigs. On this account saturation experiments tend to yield inconclusive evidence as to whether the two types of antibody combine with the same receptors of the red cells.

As has been stated, Forssman's antibody reacts *in vitro* both with watery suspensions of tissues containing the corresponding antigen and also with lipoids derived from them. But whereas watery suspensions of the tissues lead to the development of Forssman's antibody when injected into suitable animals, on the other hand the alcoholic extracts by themselves are practically devoid of this antigenic property in the strict sense. Neither do the tissue-residues which remain after thorough extraction with alcohol act as antigens. Thus the lipoids when separated from the tissues act as 'haptens', i.e. are capable of combining with the antibody *in vitro*, although they are unable to stimulate development of the latter *in vivo*. But Landsteiner and Simms (1923) demonstrated that the lipoids become activated into true antigens when added to a suitable protein, e.g. foreign serum; the antiserum which is developed in rabbits as a response to this mixture contains Forssman's antibody in addition to antibody to the foreign protein. It is essential that the lipoids and the serum should be mixed prior to injection, since simultaneous injections of the two constituents at different sites fail to produce Forssman's antibody. Pig's serum appears to be the most effective foreign protein (Heimann, 1925). Bacteria may also serve as activators when added to the lipoids (Doerr and Hallauer, 1926).

Tissue elements may possess Forssman's antigen in addition to other antigenic constituents in a state capable of stimulating antibody production. This is the case with sheep's red corpuscles; when these are injected into rabbits a response occurs to both types of antigen, and so the resulting antiserum contains a varying proportion of heterophile antibody. The latter can be removed from the serum by taking advantage of the fact that it alone is absorbed by treatment with suspensions of guinea-pig's organs, e.g. kidney, or other tissues containing Forssman's antigen. Also corpuscles, for example those of the goat, which have been saturated with heterophile antibody, when subsequently injected into rabbits develop chiefly isophile immune-body (Iijima, 1925). When boiled red cells from the sheep are injected into rabbits the resulting antiserum contains only heterophile antibody, although according to Weil and others (see Forssman and Fex, 1914) such heated corpuscles still contain antigens which are effective in producing haemolytic immune-body on injection into guinea-pigs. In rare cases, however, the red cells of individual sheep lack Forssman's antigen

and do not fix heterophile immune-body; consequently they do not undergo lysis through its influence (Mutermilch, 1924<sup>1 & 2</sup>).

Normal antibodies contained in the serum of animals of rabbit type frequently are of the heterophile variety, e.g. the natural immune-body for sheep's corpuscles in human or rabbit's serum, which is present sometimes in considerable amount (Friedemann, 1917). This may be a source of fallacy in the Wassermann test when alcoholic extracts of tissues containing Forssman's antigen are used, e.g. guinea-pig's heart. Under these circumstances a specimen of human serum which is rich in the natural antibody will lead to complement fixation in the absence of syphilitic infection (Taniguchi, 1919-20).

It is of great interest that the property of syphilitic serum to react positively in the Wassermann test shows striking analogies with Forssman's antibody (see Taniguchi, 1924). Syphilitic serum reacts with lipoid tissue constituents which are soluble in alcohol and ether but insoluble in acetone. The reaction is manifested by complement-fixation and also, under suitable conditions, by flocculation. Thus the Wassermann body, like Forssman's antibody, is lipoidophile, but the former is capable of reacting with lipoids derived from any animal species and not merely with those from animals of 'guinea-pig type'. The Wassermann body, like antibodies in general, resists heating at 55° C. There has been considerable doubt as to whether the Wassermann body represents a true antibody as Weil and Braun originally suggested, or whether it is due to some physical alteration peculiar to certain constituents (globulin) of syphilitic serum. Should the former explanation be correct, it still remains to be determined whether the Wassermann body represents an auto-antibody developed in response to lipoid-containing complexes which arise as a result of infection with *Tr. pallidum* and which act as antigens, or whether it is a natural antibody which is formed in the course of syphilis. In this connection it should be recalled that the only other example of an auto-antibody, the haemolysin found in the blood of paroxysmal haemoglobinuric patients, is also associated with syphilis; this haemolysin appears to be quite independent of the Wassermann body, however (Smith, 1923), although the opposite has been stated.

In addition there is the fact that unheated normal sera of man and various animals cause fixation of guinea-pig's complement with the Wassermann lipoids (see Mackie and Watson, 1926) and also frequently with a number of other substances, such as ethyl alcohol, peptone solution, sodium oleate, &c., but especially with peptone-cholesterol suspension (Mackie and Finkelstein, 1928). In some sera, for example those of the horse, ox, rabbit and sheep, this property is partially stable at 55° C.; in human serum and certain others it is readily destroyed by heating at this temperature. The relationship of this property to the thermo-stable Wassermann antibody is also obscure. Taniguchi (1921<sup>1</sup>) and others have observed that in the course of immunization with heterophile antigens the blood of rabbits may develop Wassermann body. Also, Sachs,

Klopstock and Weil have found that the injection into rabbits of their own tissue lipoids along with foreign serum develops a lipoidophile auto-antibody (see Weil, 1926).

In conclusion, it may be said that the question is not yet settled, but the physical properties of the Wassermann body correspond with those of immune antibodies and recent work has supplied facts which support the view that the Wassermann body is of the nature of an antibody.

#### REFERENCES.

ALTMANN, K., 1911, *Z. ImmunForsch.*, Tl. I, Orig., **8**, 24; 1912, *ibid.*, Tl. I, Orig., **13**, 219.  
 AMZEL, R., HALBER, W. & HIRSZFELD, L., 1925, *Z. ImmunForsch.*, **42**, 369.  
 ANGERER, C., 1910, *Z. ImmunForsch.*, Tl. I, Orig., **4**, 243.  
 ARRHENIUS, S., 1907, *Immunochemistry*, New York, 221.  
 BAIL, O. & MARGULIES, A., 1913, *Z. ImmunForsch.*, Tl. I, Orig., **19**, 185.  
 BORDET, J., 1898, *Ann. Inst. Pasteur*, **12**, 688; 1899, *ibid.*, **13**, 273; 1900, *ibid.*, **14**, 257; 1904, *ibid.*, **18**, 593.  
 BORDET, J. & GAY, F. P., 1906, *Ann. Inst. Pasteur*, **20**, 467.  
 BROOKS, S. C., 1919-20, *J. Med. Res.*, **41**, 399.  
 BROWNING, C. H., 1906, *Wien. klin. Wschr.*, **19**, 441.  
 BROWNING, C. H. & MACKENZIE, I., 1924, *Recent Methods in the Diagnosis and Treatment of Syphilis*, London.  
 BROWNING, C. H. & MACKIE, T. J., 1914, *Z. ImmunForsch.*, Tl. I, Orig., **21**, 422.  
 BROWNING, C. H. & SACHS, H., 1906, *Berl. klin. Wschr.*, **43**, 638, 673.  
 BROWNING, C. H. & WILSON, G. H., 1911, *J. Hyg., Camb.*, **11**, 208.  
 BUCHNER, H., 1893, *Arch. Hyg.*, Berl., **17**, 112.  
 COULTER, C. B., 1921, *J. Gen. Physiol.*, **3**, 513.  
 CROMWELL, H. W., 1922, *J. Immunol.*, **7**, 461.  
 DAREMBERG, G., 1891, *Arch. méd. exp.*, **3**, 720.  
 DOERR, R. & HALLAUER, C., 1925, *Z. ImmunForsch.*, **45**, 170; 1926, *ibid.*, **47**, 291.  
 DOERR, R. & PICK, R., 1913, *Biochem. Z.*, **50**, 129.  
 v. DUNGERN, E., 1900, *Münch. med. Wschr.*, **47**, 677.  
 DURHAM, H. E., 1900-1, *J. Exp. Med.*, **5**, 353.  
 EAGLE, H., 1929, *J. Gen. Physiol.*, **12**, 825.  
 EAGLE, H. & BREWER, G., 1929, *J. Gen. Physiol.*, **12**, 845.  
 EHRLICH, P. & MORGENROTH, J., 1899<sup>1</sup>, *Berl. klin. Wschr.*, **36**, 6; 1899<sup>2</sup>, *ibid.*, **36**, 481; 1900<sup>1</sup>, *ibid.*, **37**, 453; 1900<sup>2</sup>, *ibid.*, **37**, 681; 1901<sup>1</sup>, *ibid.*, **38**, 251; 1901<sup>2</sup>, *ibid.*, **38**, 569 and 598.  
 EHRLICH, P. & SACHS, H., 1902, *Berl. klin. Wschr.*, **39**, 492; 1905, *ibid.*, **42**, 556.  
 v. EISLER, M., 1909, *Z. ImmunForsch.*, Tl. I, Orig., **2**, 159.  
 FORSSMAN, J., 1911, *Biochem. Z.*, **37**, 78; 1928, *Handb. d. path. Mikroorg.*, 3rd ed., **8**, 469.  
 FORSSMAN, J. & FEX, J., 1914, *Biochem. Z.*, **61**, 6.  
 FRAENKEL, E., 1911, *Z. ImmunForsch.*, Tl. I, Orig., **10**, 388.  
 FRIEDBERGER, E. & BEZZOLA, C., 1908, *Zbl. Bakt.*, Abt. I, Orig., **46**, 412.  
 FRIEDBERGER, E. & MORESCHI, C., 1908, *Zbl. Bakt.*, Abt. I, Orig., **45**, 346.  
 FRIEDBERGER, E. & SCHIFF, F., 1913, *Berl. klin. Wschr.*, **50**, 1557, 2328.  
 FRIEDBERGER, E. & SEELIG, A., 1908, *Zbl. Bakt.*, Abt. I, Orig., **46**, 421.  
 FRIEDE, K. A., 1925, *Zbl. Bakt.*, Abt. I, Orig., **96**, 154.  
 FRIEDE, K. A. & GRÜNBAUM, F. T., 1925, *Z. ImmunForsch.*, **44**, 314.  
 FRIEDEMANN, U., 1917, *Biochem. Z.*, **80**, 333.  
 GEORGI, W., 1919, *Arb. Inst. exp. Ther.*, Frankfurt, Heft **9**, 33.  
 HADDA, S. & ROSENTHAL, F., 1913, *Z. ImmunForsch.*, Tl. I, Orig., **16**, 524.  
 HEIMANN, F., 1925, *Z. ImmunForsch.*, **44**, 44, 523; 1927, *ibid.*, **50**, 525.  
 HEKTOEN, L., 1927, *J. Immunol.*, **14**, 1.  
 HEKTOEN, L. & SCHULHOFF, K., 1922, *J. Infect. Dis.*, **31**, 32; 1923, *ibid.*, **33**, 224.  
 HILL, A. & PARKER, G., 1925, *J. Path. Bact.*, **28**, 1.  
 HIRSZFELD, L. & HALBER, W., 1923, *C.R. Soc. Biol.*, Paris, **89**, 1370.

HUNTOON, F. M., 1921, *J. Immunol.*, **6**, 117.  
 HUNTOON, F. M. & ETRIS, S., 1921, *J. Immunol.*, **6**, 123.  
 HYDE, R. R. & PARSONS, E. I., 1927, *Amer. J. Hyg.*, **7**, 11.  
 IIJIMA, T., 1923, *J. Path. Bact.*, **28**, 519; 1925, *ibid.*, **28**, 397.  
 IWAI, S., 1917, *Mitt. med. Fak.*, Kyushu, **4**, 139.  
 KAHN, R. L. & LYON, D. S., 1921, *J. Infect. Dis.*, **29**, 651.  
 KOSAKAI, M., 1918, *J. Immunol.*, **3**, 109; 1919-20, *J. Path. Bact.*, **23**, 425.  
 KRITSCHEWSKY, J. L., 1923, *Z. ImmunForsch.*, Tl. I, Orig., **36**, 1.  
 LANDSTEINER, K. & JAGIĆ, N., 1904, *Münch. med. Wschr.*, **51**, 1185.  
 LANDSTEINER, K. & LEVENE, P. A., 1926-7, *Proc. Soc. Exp. Biol.*, N.Y., **24**, 693.  
 LANDSTEINER, K. & PRASEK, E., 1912, *Z. ImmunForsch.*, Tl. I, Orig., **13**, 403.  
 LANDSTEINER, K. & ROCK, H., 1912, *Z. ImmunForsch.*, Tl. I, Orig., **14**, 14.  
 LANDSTEINER, K. & VAN DER SCHEER, J., 1925<sup>1</sup>, *J. Exp. Med.*, **41**, 427; 1925<sup>2</sup>, *ibid.*, **42**, 123.  
 LANDSTEINER, K. & SIMMS, S., 1923, *J. Exp. Med.*, **38**, 127.  
 LANDSTEINER, K. & WELECKI, St., 1910, *Z. ImmunForsch.*, Tl. I, Orig., **8**, 397.  
 v. LIEBERMANN, L. & v. FENYVESSY, B., 1909, *Z. ImmunForsch.*, Tl. I, Orig., **2**, 431; 1911, *ibid.*, Tl. I, Orig., **10**, 479.  
 LIEFMANN, H. & ANDREEW, 1911, *Z. ImmunForsch.*, Tl. I, Orig., **11**, 707.  
 LOCKE, A. & HIRSCH, E. F., 1924, *J. Infect. Dis.*, **35**, 519; 1925, *ibid.*, **37**, 449.  
 LOCKE, A., MAIN, E. R. & HIRSCH, E. F., 1926, *J. Infect. Dis.*, **39**, 126.  
 MCKENDRICK, A. G., 1910-11, *Proc. Roy. Soc.*, B, **83**, 493.  
 MACKIE, T. J., 1925, *J. Hyg.*, Camb., **24**, 176.  
 MACKIE, T. J. & FINKELSTEIN, M. H., 1928, *J. Hyg.*, Camb., **28**, 172.  
 MACKIE, T. J. & WATSON, H. F., 1926, *J. Hyg.*, Camb., **25**, 176.  
 MANWARING, W. H., 1905, *J. Infect. Dis.*, **2**, 460, 485; 1907, *Zbl. Bakt.*, Abt. I, Orig., **43**, 820; 1908, *ibid.*, **45**, 55.  
 MARKL, 1902, *Z. Hyg. InfektKr.*, **39**, 86.  
 MEYERSTEIN, A., 1923, *Z. ImmunForsch.*, Tl. I, Orig., **38**, 403.  
 MORGENTHROTH, J., 1903, *Münch. med. Wschr.*, **50**, 61.  
 MORGENTHROTH, J. & BIELING, R., 1915, *Biochem. Z.*, **68**, 85.  
 MORGENTHROTH, J. & ROSENTHAL, F., 1911, *Biochem. Z.*, **36**, 190; 1912, *ibid.*, **39**, 88.  
 MORGENTHROTH, J. & SACHS, H., 1902, *Berl. klin. Wschr.*, **39**, 631.  
 MUDD, S. & MUDD, E. B. H., 1926, *J. Exp. Med.*, **43**, 127.  
 MUIR, R., 1903, *Lancet*, Lond., ii, 446; 1911-12, *J. Path. Bact.*, **16**, 143, 523.  
 MUIR, R. & BROWNING, C. H., 1904, *Proc. Roy. Soc.*, **74**, 298; 1906, *J. Hyg.*, Camb., **6**, 1; 1909, *J. Path. Bact.*, **13**, 232.  
 MUIR, R. & FERGUSON, A. R., 1906, *J. Path. Bact.*, **11**, 84.  
 MUIR, R. & MCNEE, J. W., 1912-13, *J. Path. Bact.*, **17**, 92.  
 MÜLLER, P. T., 1908, *Arch. Hyg.*, Berl., **64**, 62.  
 MUTERMILCH, S., 1924<sup>1</sup>, *C.R. Acad. Sci.*, Paris, **178**, 2134, 2285; 1924<sup>2</sup>, *Ann. Inst. Pasteur*, **38**, 1002.  
 NATHAN, E., 1913, *Z. ImmunForsch.*, Tl. I, Orig., **19**, 216.  
 NEUFELD, F., 1908, *Arb. Gesundh.Amt.*, Berl., **28**, 125.  
 NOGUCHI, H., 1910, *The Serum Diagnosis of Syphilis*, Philadelphia.  
 ORUDSCHIEW, D., 1913, *Z. ImmunForsch.*, Tl. I, Orig., **16**, 268.  
 OTTENBERG, R., KALISKI, D. J. & FRIEDMAN, S. S., 1913, *J. Med. Res.*, **28**, 141.  
 PHILOSOPHOW, P., 1909, *Biochem. Z.*, **20**, 292.  
 v. POGGENPOHL, S., 1909, *Biochem. Z.*, **22**, 64.  
 POWELL, H. M., 1926, *J. Immunol.*, **12**, 1.  
 REINER, L. & FISCHER, O., 1929, *Z. ImmunForsch.*, **61**, 317.  
 RONDONI, P., 1910, *Z. ImmunForsch.*, Tl. I, Orig., **7**, 515.  
 RUBINSTEIN, P. L., 1927, *Z. ImmunForsch.*, **50**, 114.  
 SACHS, H., 1902, *Berl. klin. Wschr.*, **39**, 181, 216; 1929, *Handb. d. path. Mikroorg.*, hrsg. Kolle, Kraus u. Uhlenhuth, Jena, **2**, 880.  
 SACHS, H. & NATHAN, E., 1913, *Z. ImmunForsch.*, Tl. I, Orig., **19**, 235.  
 SACHS, H. & TERUUCHI, Y., 1907, *Berl. klin. Wschr.*, **44**, 467.  
 SCHELLER, R., 1910, *Zbl. Bakt.*, Abt. I, Orig., **56**, 120.  
 SCHIFF, F. & ADELSSBERGER, L., 1924, *Z. ImmunForsch.*, **40**, 335.  
 SCHULTZ, W., 1905, *Deuts. Arch. klin. Med.*, **84**, 552.  
 SMITH, R. P., 1923, *J. Path. Bact.*, **28**, 196.

TANIGUCHI, T., 1919-20, *J. Path. Bact.*, **23**, 368; 1921<sup>1</sup>, *ibid.*, **24**, 122; 1921<sup>2</sup>, *ibid.*, **24**, 217, 241, 356, 456; 1922, *ibid.*, **25**, 77; 1924, see Browning, C.H. and Mackenzie, I., 1924.

THIELE, F. H. & EMBLETON, D., 1914, *Z. ImmunForsch.*, Tl. I, Orig., **20**, 1; 1914-15, *J. Path. Bact.*, **19**, 372.

TODD, C., 1930, *Proc. Roy. Soc.*, B, **106**, 20.

TODD, C. & WHITE, R. G., 1910, *J. Hyg., Camb.*, **10**, 185; 1911-12, *Proc. Roy. Soc.*, B, **84**, 255.

TOPLEY, W. W., 1915, *Proc. Roy. Soc.*, B, **88**, 396.

TROU-HIA-HSÜ, 1922, *Z. ImmunForsch.*, Tl. I, Orig., **34**, 507.

UNGERMANN, E. & KANDIBA, L., 1912, *Arb. GesundhAmt.*, Berl., **40**, 24.

WEIL, A. J., 1926, *Z. ImmunForsch.*, **46**, 81.

WITEBSKY, E., 1927, *Z. ImmunForsch.*, **51**, 161.

WRIGHT, H. D. & MACCALLUM, P., 1922, *J. Path. Bact.*, **25**, 316.

YASUI, K., 1929<sup>1</sup>, *Z. ImmunForsch.*, **63**, 215; 1929<sup>2</sup>, *ibid.*, **63**, 440.

YOSHINARE, N., 1922, *J. Path. Bact.*, **25**, 153.

ZADE, M., 1909, *Z. ImmunForsch.*, Tl. I, Orig., **2**, 81.

### Cytolysins.

BY S. P. BEDSON.

It is hardly surprising that the work of Bordet (1898) on the production of hæmolysins should have had as its immediate result the endeavour to produce similar sera for other kinds of cells. Quite apart from the purely academic interest attaching to the study of the immune response to a variety of cellular antigens this work had several practical objectives. It was thought that by means of specific sera prepared against the cells and tissues of the body light might be thrown on many physiological and pathological processes and further that such sera might be used therapeutically. It was believed that certain conditions, such as nephritis or diabetes, for instance, might be caused by the auto-production of cytolysins, and to verify this hypothesis the attempt was made to reproduce these pathological states in animals by means of the appropriate cytolytic sera. Should this hypothesis have proved correct then the next step would have been to attempt treatment by means of specific anti-cytolysins. It was also hoped that specific cytolytic sera might prove useful in the treatment of certain types of goitre, the leukæmias, &c.

The conception was an ingenious and attractive one, and though the early work in this field promised well, subsequent investigations failed to confirm it, and this line of approach has been largely abandoned. This failure was in large measure due to the fact that it is extremely difficult, if not impossible in the majority of cases, to prepare a cytolytic serum possessed of a clean-cut organ-specificity. Even had success been achieved it is extremely improbable that anticytolysins would have been of any value therapeutically, for it is doubtful if such things as anti-antibodies do in reality exist. Nevertheless, it would be wrong to give the impression that this line of investigation has been entirely fruitless. It was by means of a cytolytic serum—one prepared against the mammalian platelet—that Ledingham (1914) was able to reproduce purpura hæmorrhagica or essential thrombopenia experimentally in animals, thus adding a brilliant

experimental proof to the work of Duke (1912) on the part played by the platelet in the hæmorrhagic diathesis. Further, paroxysmal hæmoglobinuria has been shown to be due to an autohæmolysin (Donath and Landsteiner, 1906), and cold urticaria, which is sometimes associated with the former condition, would also appear to be due to a cytolysin (Harris, Lewis and Vaughan, 1928). The latter workers refer to this cytotoxin as a dermolysin, but since the damage must be to endothelium it would be more correct to speak of it as an endotheliolysin. Finally, the work of Lumsden (1925<sup>1 & 2</sup>, 1926, 1927) on cytolytic sera for malignant cells is throwing light on the problem of immunity in malignant disease and leading possibly to results of great therapeutic importance.

Other uses have been found for cytolytic sera. They have been employed in the study of the genesis and interrelationship of the different cells of the body as a control to the purely morphological study of this problem. It is in the realms of haematology that this use of cytolytic sera has found its chief application.

#### NATURE OF CYTOLYSINS.

Cytolytic sera contain antibodies which are susceptible of demonstration in the test-tube by any of the usual immunity reactions—precipitation, agglutination, complement fixation and lysis. Like the bacteriolysins and hæmolysins they are thermo-stable and for their lytic action they require the presence of complement. The lytic action of these sera *in vitro* is not nearly so dramatic or complete as is the case of the lysis of red corpuscles by a hæmolytic serum, and for this reason the custom has arisen of describing the sera prepared against tissue cells as cytotoxins rather than cytolsins. This is perhaps unfortunate, for it suggests that there is some inherent difference between hæmolysins and the other anticell sera, whereas there is no good reason for making this assumption. The difference is one of antigens. The red corpuscles, in virtue of their structure, readily lend themselves to the demonstration of lytic action, the other tissue cells do not. It has been observed that the lytic action of an anti-epithelial serum was much more complete if the reaction were carried out in the peritoneal cavity of an immune animal as in the Pfeiffer reaction (von Dungern, 1899) and the same was found to be true for spermotoxins (Moxter, 1900). Many normal sera are lytic or toxic for cells of another species, the serum of the eel is a good example of this, and, broadly speaking, the term cytolysin should include these as well as other cell poisons such as the snake venoms. It is impossible, however, to do more than mention these here, and for the purpose of this article the term cytolysin will be used in its more restricted sense.

#### SPECIFICITY OF CYTOLYSINS.

One not infrequently meets the statement that this class of antiserum is not strictly specific in its action, and although a superficial acquaintance with the subject might lead to such a conclusion, it is erroneous. All

those antisera which have been prepared against pure cell antigens, such as anti-epithelial, anti-sperm and anti-platelet sera, are cell-specific, but where the antigen consisted of an organ emulsion the antiserum obtained is not organ-specific. This is not surprising, for organs comprise a variety of cells, some of which are peculiar to a certain organ, while others again, such as connective tissue cells, muscle cells and vascular endothelium, are common to all. One would, therefore, expect that an anti-organ serum would contain a variety of antibodies, one specific to the organ, and others common to the majority of tissues from the same animal species. That such is in reality the case emerges from the work of Fleisher, Hall and Arnstein (1920, 1921). It has been claimed (Beebe, 1905) that the antisera obtained with the nucleoproteins of organs are more specific than those produced with whole organ emulsions, but this has failed to find confirmation (Pearce and Jackson, 1906; Pearce, Karsner and Eisenbrey, 1911; Wells, 1913). It is an interesting fact that, almost without exception, cytolytic sera are hæmolytic, though in varying degree. In the case of anti-organ sera this might be thought to be due to the presence of red corpuscles in the tissues, but since it occurs in the case of sera prepared with emulsions of organs which have been carefully washed through to free them from red cells, and also in anti-sperm and anti-platelet sera, where the antigen can be shown to be free from red corpuscles by microscopic examination, this cannot be the reason. The most probable explanation is that the hæmolysin is in the nature of a group antibody, a phenomenon we are familiar with in the case of antisera to closely allied bacteria. This finds support in the observation that an anti-platelet serum when absorbed with platelets loses both red cell and platelet antibodies, but when absorbed with red cells the hæmolytic antibody is alone removed (Sacerdotti, 1908; Bedson, 1921). It might be mentioned here that Davide (1925) has shown that an anti-fibrinogen serum is strongly hæmolytic, and that absorption with either red corpuscles or fibrinogen removes both antibodies. In consequence of this Davide postulates a possible origin of fibrinogen from the breakdown of red corpuscles. Whatever may be the explanation of the hæmolytic activity of cytolytic sera, the fact is of considerable importance, for as Pearce has shown (1904, 1906), some of the changes which have been produced *in vivo* by means of these sera have been due to the red cell antibody and not to the specific one. Cytolytic sera are characterized by a species specificity with possibly some slight overlapping between closely allied species, and it would appear that the tissue proteins are stamped as it were with the imprint of the species. There are, however, exceptions to this, such as the protein of the lens of the eye, which is immunologically common to a variety of species. Perhaps this explains why this protein is sufficiently foreign when introduced parenterally to incite the production of iso- and even auto-antibodies. The interesting work of recent years on the chemistry of bacterial antigens naturally suggests that the specificity of cell antigens within a species may be determined by non-protein and

non-antigenic substances. There is some evidence that lipoids may do this. Sachs and Klopstock (1925) have shown that cholesterol or lecithin mixed with a foreign serum form antigens which produce sera reacting specifically with these lipoids. Further, Brandt, Guth and Müller (1926) claim that brain lipoids can be differentiated from other tissue lipoids, since they modify specifically antigens to which they are linked. Additional evidence of the role played by lipoids in determining the specificity of cell antigens emerges from the work of Landsteiner and van der Scheer (1925, 1926) on the antigens of the red corpuscles. This opens up an interesting field for further research.

#### ISOCYTOLYSINS.

The fact that cytolytic sera are so strictly species-specific would rather lead one to expect that it would not be possible to produce isocytolysins, but there is evidence that this can be achieved. The classical experiments of Ehrlich and Morgenroth (1900) on the production of isohæmolysins in the goat are well known, and the question of isohæmolysins has been further advanced by the work of Todd and White (1910) on the production of isolysins for the red corpuscles of the ox. It must not be forgotten, however, that the red corpuscle exhibits antigenic types within a species. There seems no doubt that Metchnikoff (1899) produced an isolysin for guinea-pig spermatozoa, and the case of lens protein has been already referred to. The other instances of isocytolysins met with in the literature are of a somewhat doubtful nature. Hulot and Ramond (1901) noted changes in the liver and kidneys of guinea-pigs which had received a number of injections of guinea-pig liver and kidney respectively, but their experiments were few in number and not at all convincing. Néfedieff (1901) attempted to produce an isonephrolysin in the rabbit by ligaturing the ureter on one side and collecting blood after the lapse of several weeks. This serum when inoculated into normal rabbits was stated to produce albuminuria and degenerative changes in the kidney, but here again the evidence does not carry conviction.

#### AUTOCYTOLYSINS.

In view of the attempt to explain certain forms of disease on the basis of an autoproduction of antibodies to tissue cells it is only natural that an endeavour should have been made to put this to experimental proof. Bierry (1902) claims that the tying of the renal pedicle—with the exception of the ureter—on one side in dogs gave rise to albuminuria, and Castaigne and Rathery (1902), employing the same operative procedure in rabbits, observed the degenerative changes in the other kidney. Neither of these pieces of work is at all convincing, and, except for the case of auto-antibodies for lens protein, it would seem doubtful whether autocytolysins have been produced. Todd and White (1910) failed to produce an autolysin to ox red corpuscles.

## ANTICYTOLYSINS.

Since it was thought that some pathological states were the work of autolysins, the attempt to produce anticytolsins was a logical step in the direction of scientific therapeutics. There is no doubt that if one immunizes an animal with a cytolytic serum one will obtain a serum endowed with neutralizing properties, provided that the animal employed belongs to a different species to the one in which the cytolytic serum was made. At first sight it would seem that an anti-antibody had been produced and this erroneous interpretation was for long the accepted one. The antibody in a cytolytic serum—as in any antiserum for that matter—is intimately associated with the serum proteins and an anticytolytic serum is nothing more than an antiserum to these proteins: a precipitin in other words. The complex resulting from the union of the serum proteins of the cytolytic serum with the precipitin in the so-called anticytolytic serum carries with it the cytolytic antibody, which is thus prevented from acting either *in vivo* or *in vitro*. That this is so emerges from experiments with anti-platelet and allied sera (Bedson, 1922; Johnston, 1924).

## REVIEW OF THE VARIOUS CYTOLYTIC SERA WHICH HAVE BEEN STUDIED.

*Anticell Sera.*

*Against epithelium.* Von Dungern (1899) prepared an antiserum to the ciliated epithelium of the trachea of the ox in guinea-pigs. Its lytic action was not very obvious *in vitro*, but could be readily demonstrated if the epithelial cells were introduced into the peritoneal cavity of the immunized guinea-pig. This serum was slightly haemolytic, but an anti-serum to ox red cells was without effect on epithelium, and an anti-epithelium serum showed a greater affinity for epithelium than for red corpuscles, for when placed in the presence of both cells it lysed epithelium but was without action on the red corpuscles.

*Against spermatozoa.* This serum was prepared independently by Landsteiner (1899) and Metchnikoff (1900). *In vitro* it was not obviously lytic, but showed its toxic action by immobilizing the spermatozoa. Moxter (1900) immunized rabbits with sheep spermatozoa and found that lysis could be readily demonstrated if the spermatozoa were injected into the peritoneal cavity of the immunized rabbit. Anti-spermatozoa sera contain agglutinins and lysins for red corpuscles.

*Against leucocytes.* Metchnikoff (1899) was the first to prepare a serum which was active against leucocytes by inoculating guinea-pigs with suspensions of rat's spleen. As one would expect, this serum was lytic for all types of leucocytes. Metchnikoff (1899) also immunized guinea-pigs with emulsions of mesenteric lymph glands of the rabbit. This serum was also found to act indiscriminately on all leucocytes—mononuclear and polymorphonuclear—an observation which was repeated by Besredka (1900). More recently, however, Bedson and Johnston

(1925) found that an anti-lymph-gland serum behaved with considerable cell specificity when tested *in vivo*. It produced a marked fall in the total leucocyte count, which was almost entirely at the expense of the mononuclear leucocytes (large mononuclears and lymphocytes). This remarkable cell-specificity of a leucolytic serum finds support in the observations made with sera prepared against polymorphonuclear leucocytes. Ledingham and Bedson (1915) prepared a serum against guinea-pig polymorphonuclears, the leucocytes being obtained by producing a peritoneal exudate in guinea-pigs by injecting sterile broth into the peritoneal cavity. When collected some five hours after the introduction of the broth, this exudate was found to consist almost entirely of polymorphonuclear leucocytes. The serum, which was prepared in rabbits, was found to be highly toxic for guinea-pigs and produced a most dramatic, though temporary, drop in the leucocyte count. Differential counts and examinations of blood films showed that this was due to the destruction of polymorphonuclears, which had almost entirely disappeared from the circulation. The specificity of a polymorphonuclear antiserum finds confirmation in subsequent work (Lindstroem, 1921; Bedson, 1922). The action of an anti-leucocyte serum can be demonstrated *in vitro* by lysis, agglutination or the precipitin reaction. Lysis does take place, but is not nearly so complete as in the case of lysis of red corpuscles by an appropriate serum. Macroscopically the reaction does not result in any change which is clearly obvious, but observed under the microscope the cytoplasm of the leucocytes can be seen to swell up and eventually disintegrate. Agglutination is not satisfactory because of the tendency of leucocyte suspension to flocculate spontaneously. Perhaps the precipitin test carried out with an extract of leucocytes is the most satisfactory of test-tube reactions (Menne, 1922). Anti-leucocyte sera all possess red cell antibody in greater or less quantity, however carefully the antigen may have been prepared to ensure freedom from red cells. Attempts have been made to treat leukæmia with anti-leucocyte sera (Lindstroem, 1921) but only a transitory improvement has been obtained.

*Against platelets.* Although Marino (1905) was the first to prepare an antiserum to this element of the blood, the study of this serum dates from the investigations of Le Sourd and Pagnier, who were the first to devise a method for obtaining platelets in purity (1906<sup>1</sup>), which enabled them to prepare a satisfactory serum. They showed that an anti-platelet serum lysed platelets *in vitro*, and that in the animal it caused a disappearance of the platelets from the circulation without having much effect on the other formed elements of the blood (1906<sup>2&3</sup>, 1907). These observations suggested that an anti-platelet serum contained a specific antibody, but it was not until some years later that conclusive proof was forthcoming. In 1914 Ledingham made the fundamental observation that an anti-platelet serum was capable of evoking a typical hæmorrhagic purpura in the animal, and this opened the way for a series of researches

(Ledingham and Bedson, 1915 ; Bedson, 1921, 1922, 1923 ; Bedson and Johnston, 1925), which have definitely established that this serum contains an antibody specific for platelets, and that the platelet is antigenically distinct from all other elements of the blood (red corpuscles, leucocytes and plasma proteins). This serum agglutinates and lyses platelets *in vitro*, and in the animal it causes the platelets to disappear from the circulation and produces purpura. The latter effect is only obtained if a sufficient dose of serum is employed and is invariably associated with great prolongation of the bleeding time. The coagulation time shows little or no change as in essential thrombopenia in man. Smaller doses of the serum result only in destruction of platelets, and, by grading the dose, one can get anything from a slight reduction in the platelet count up to complete destruction of platelets without purpura. This clearly demonstrates that the purpura is not solely due to the removal of the platelets, a fact which has been demonstrated experimentally by other means (Bedson, 1922 ; Roskam, 1921). For the production of purpura some other factor is necessary and this would appear to be damage to the capillary endothelium (Bedson, 1922). It would seem, then, that anti-platelet serum is capable of acting both on platelets and capillary endothelium, which is not surprising in view of a probable common ancestry, though it naturally shows a preference for the specific antigen and therefore attacks platelets first. Anti-platelet sera are invariably haemagglutinating and haemolytic, and, since absorption with red corpuscles removes this antibody but not the specific one for platelets, and absorption with platelets reduces the two equally (Bedson, 1922), one may regard the development of red cell antibody in response to a platelet antigen as pertaining to the phenomenon of group antibodies. Anti-platelet sera, as well as being cell-specific, show a strict species-specificity. It might be thought that in this serum one had a ready means for settling the vexed question of platelet genesis, and although it has enabled one to dispose of the various hypotheses which derived the platelet from one or other of the blood elements and to show that it is produced in the bone-marrow (Bedson and Johnston, 1925), beyond that it has not been possible to go. Whether the platelet arises in the bone-marrow along a separate genetic line or is produced by the megakaryocytes awaits experimental determination. It is of significance that Ledingham and Woodcock (1921) were able to show that sera prepared against the thrombocyte of the bird and the spindle cell of the frog—cells analogous with the mammalian platelet—were capable of producing purpura. The bearing of these findings on the ætiology of essential thrombopenia would appear obvious, but attempts to demonstrate thrombocytolytic activities in the serum of cases of that condition have given negative results (unpublished work). In addition to this it is difficult to understand why splenectomy should provide a cure, as it apparently does in a large percentage of cases, unless it be that the spleen itself is the centre of production of a platelet toxin as well as being the grave of those elements when they are damaged.

In the animal splenectomy merely protects against the purpurigenic action of anti-platelet serum in virtue of the enormous increase in the number of circulating platelets which it gives rise to, and as soon as the platelet count returns to normal after splenectomy—which in the rabbit and guinea-pig occurs in a few weeks—the animal reacts both qualitatively and quantitatively to anti-platelet serum in the same manner as normal unsplenectomized controls (Bedson, 1924). There is much that requires elucidation before the ætiology of thrombopenic purpura will be correctly understood.

*Against the cells of malignant growth.* Attempts have been made from time to time to prepare antisera to malignant tissues, but conflicting results have been obtained. Lambert (1914), for instance, immunized guinea-pigs with an emulsion of a rat sarcoma, and tested for cytolytic action by growing the tissues in the plasma of the immunized guinea-pigs. He failed to find any evidence of a specific cytolysin for the rat sarcoma cells, and stated that he only obtained species-specific antibodies. Lumsden (1925<sup>1</sup>, 1926), however, has produced definite evidence that sera containing cancer antibodies can be obtained. Like Lambert, he made use of the tissue culture technique for the demonstration of these antibodies, and has carried out the major part of his work with the Jensen rat sarcoma, a strain of mouse carcinoma (M 63 of the Imperial Cancer Research) and a human mammary carcinoma. The sera were prepared by repeated inoculations of tumour suspensions into animals of a heterologous species (rabbits and sheep), and though, of course, the sera contained antibodies which were merely species-specific, they also possessed others, specific for the malignant cells. Tumour cells grown in the heterologous anti-cancer serum rapidly died and disintegrated, whereas in the control cultures made with normal rabbit serum they survived and grew well. The anti-malignant activities of these sera would not appear to be tumour-specific but to be active against malignant cells generally. Thus, a serum prepared with a human breast carcinoma was toxic for the cells of M 63 and the Jensen rat sarcoma, whilst being innocuous to normal mouse and rat tissues (Lumsden, 1926). In this respect it is interesting to note that Lumsden found that a serum prepared against the spermatozoa of a higher mammal killed mouse cancer cells rapidly, but had only a slight action on mouse kidney, and none at all on mouse fibroblasts or heart-muscle.

The specific cancer cytolysin in these sera requires the co-operation of complement for its action *in vitro*, and unlike the anti-species cytolysins contained in these sera, it is thermo-labile, being destroyed in 6 to 7 hours at 50° C. (Kohn-Speyer, 1927). In the animal the specific cytolytic activity could only be demonstrated by injection into or around a tumour and at the same time temporarily stopping the circulation to the tumour-bearing part (Lumsden, 1925<sup>2</sup>; Lumsden and Stephens, 1927). Applied in this way, Lumsden has been able to make implanted tumours (Jensen rat sarcoma) on the feet of rats regress and disappear, and when this

was done the animals were subsequently found to be immune. Strange to say, the serum of such an immune rat was not found to be lytic for the cells of the Jensen rat sarcoma *in vitro*, and it did not even become so after hyperimmunization with the homologous tumour (Lumsden, 1926). There is evidence (Lumsden, 1927), however, that the serum does contain specific lethal antibodies, but that they require the addition of an active principle elaborated by the leucocytes of the immune animal for their demonstration. Neither of these two factors alone is active.

So far the work of Lumsden has been carried out with implanted tumours only ; it remains to be seen whether his results hold good for spontaneous malignant growths. Attempts have been made from time to time to develop a diagnostic test for malignant disease based on an immunity reaction. The indifference of the tissues of the tumour-bearing animal to the malignant cells in the great majority of cases is not a hopeful augury of such an aim being achieved, and the complement-fixation reaction has given conflicting and unsatisfactory results. The reaction of Freund and Kaminer (1910), which is based on their demonstration that the serum of people suffering from malignant disease has the power of neutralizing the carcinolytic action of normal human serum, has not found favour. Many flocculation tests have been evolved, some of which are founded on a frankly non-specific basis, but a consideration of these is outside the scope of this article.

#### *Anti-Organ Sera.*

In the decade following the work of Bordet on the production of specific hæmolsins, attempts were made to prepare antisera to practically every organ of the body. The hope that these sera would prove organ-specific, and so furnish a convenient method of investigating experimentally the functions of organs and also the possibility of reproducing certain forms of disease, have not been fulfilled. The history of all these sera is the same ; sweeping claims as to organ-specificity *in vivo* by the first to make the serum have been followed by more sober-minded and critical work showing how ill-founded the original claims were. In an earlier part of this article the reasons for not expecting these sera to be organ-specific have been discussed, and there is no need to recapitulate them here. Attention was also drawn to the more recent work of Fleisher, Hall and Arnstein (1920, 1921) showing that by carefully conducted *in-vitro* experiments anti-organ sera can be shown to possess some organ-specific antibody. It seems hardly necessary in view of the above remarks to refer to these different sera individually, and those interested are referred to a paper by Ritchie (1908) on adrenolytic and thymolytic sera, where a good bibliography will be found. The syncytolytic sera perhaps deserve mention because the conception of an autosyncytolysin in pregnancy has been made the starting-point of numerous attempts to elaborate a specific diagnostic test for pregnancy, of which the Abderhalden reaction is the latest development. It is supposed that the cells of the

chorionic villi become detached and invade the maternal circulation with the result that the production of specific antibodies is excited (Scholten and Veit, 1903). Conflicting results have been obtained in attempting to verify this hypothesis, and there is considerable doubt as to the specificity of these reactions.

## REFERENCES.

BEDSON, S. P., 1921, *J. Path. Bact.*, **24**, 469; 1922, *ibid.*, **25**, 94; 1923, *ibid.*, **26**, 145; 1924, *Lancet*, Lond., ii, 1117.  
 BEDSON, S. P. & JOHNSTON, M. E., 1925, *J. Path. Bact.*, **28**, 101.  
 BEEBE, S. P., 1905, *J. Exp. Med.*, **7**, 733.  
 BESREDKA, 1900, *Ann. Inst. Pasteur*, **14**, 390.  
 BIERRY, H., 1902, *C.R. Soc. Biol.*, Paris, **54**, 1003.  
 BORDET, J., 1898, *Ann. Inst. Pasteur*, **12**, 688.  
 BRANDT, R., GUTH, H. & MÜLLER, R., 1926, *Klin. Wschr.*, **5**, 655.  
 CASTAIGNE, J. & RATHERY, F., 1902, *C.R. Soc. Biol.*, Paris, **54**, 563.  
 DAVIDE, H., 1925, *Acta med. scand.*, Suppl. **13**.  
 DONATH, J. & LANDSTEINER, K., 1906, *Z. klin. Med.*, **58**, 173.  
 DUKE, W. W., 1912, *Arch. Intern. Med.*, **10**, 445.  
 VON DUNGERN, 1899, *Münch. med. Wschr.*, **46**, 1228.  
 EHRLICH, P. & MORGENROTH, J., 1900, *Berl. klin. Wschr.*, **37**, 453.  
 FLEISHER, M. S., HALL, T. G. & ARNSTEIN, N., 1920, *J. Immunol.*, **5**, 437; 1921, *ibid.*, **8**, 223.  
 FREUND, E. & KAMINER, G., 1910, *Biochem. Z.*, **26**, 321.  
 HARRIS, K. E., LEWIS, T. & VAUGHAN, J. M., 1928, *Brit. Med. J.*, ii, 885.  
 HULOT, J. & RAMOND, F., 1901, *C.R. Soc. Biol.*, Paris, **53**, 1133.  
 JOHNSTON, M. E., 1924, *Brit. J. Exp. Path.*, **5**, 261.  
 KOHN-SPEYER, A. C., 1927, *Lancet*, Lond., ii, 377.  
 LAMBERT, R. A., 1914, *J. Exp. Med.*, **19**, 277.  
 LANDSTEINER, K., 1899, *Zbl. Bakter.*, Abt. I, **25**, 546.  
 LANDSTEINER, K. & VAN DER SCHEER, J., 1925, *J. Exp. Med.*, **41**, 427; 1926, *ibid.*, **42**, 123.  
 LEDINGHAM, J. C. G., 1914, *Lancet*, Lond., i, 1673.  
 LEDINGHAM, J. C. G. & BEDSON, S. P., 1915, *Lancet*, Lond., i, 311.  
 LEDINGHAM, J. C. G. & WOODCOCK, H. M., 1921, *J. Path. Bact.*, **24**, 365.  
 LINDSTROEM, G., 1921, *C.R. Soc. Biol.*, Paris, **84**, 17.  
 LUMSDEN, T., 1925<sup>1</sup>, *Lancet*, Lond., i, 383; 1925<sup>2</sup>, *ibid.*, ii, 539; 1926, *ibid.*, ii, 112; 1927, *ibid.*, i, 116.  
 LUMSDEN, T. & STEPHENS, J. G., 1927, *Lancet*, Lond., ii, 375.  
 MARINO, M. F., 1905, *C.R. Soc. Biol.*, Paris, **58**, 194.  
 MENNE, F. R., 1922, *J. Infect. Dis.*, **31**, 455.  
 METCHNIKOFF, E., 1899, *Ann. Inst. Pasteur*, **13**, 737; 1900, *ibid.*, **14**, 1.  
 MOXTER, 1900, *Deuts. med. Wschr.*, **26**, 61.  
 NÉFEDIEFF, N., 1901, *Ann. Inst. Pasteur*, **15**, 17.  
 PEARCE, R. M., 1904, *J. Med. Res.*, **12**, 329; 1906, *ibid.*, **14**, 541.  
 PEARCE, R. M. & JACKSON, H. C., 1906, *J. Infect. Dis.*, **3**, 742.  
 PEARCE, R. M., KARSNER, H. T. & EISENBREY, A. B., 1911, *J. Exp. Med.*, **14**, 44.  
 RITCHIE, W. T., 1908, *J. Path. Bact.*, **12**, 140.  
 ROSKAM, J., 1921, *C.R. Soc. Biol.*, Paris, **84**, 844.  
 SACERDOTTI, C., 1908, *Arch. Sci. med.*, **32**, 339.  
 SACHS, H. & KLOPSTOCK, A., 1925, *Biochem. Z.*, **159**, 491.  
 SCHOLLEN, R. & VEIT, J., 1903, *Z. Geburtsh. Gynäk.*, **49**, 210.  
 LE SOURD, L. & PAGNIEZ, P., 1906<sup>1</sup>, *C.R. Acad. Sci., Paris*, **142**, 1562; 1906<sup>2</sup>, *C.R. Soc. Biol.*, Paris, **61**, 109; 1906<sup>3</sup>, *ibid.*, **61**, 562; 1907, *ibid.*, **63**, 561.  
 TODD, C. & WHITE, 1910, *J. Hyg., Camb.*, **10**, 185.  
 WELLS, H. G., 1913, *Z. ImmunForsch.*, Tl. I, Orig., **19**, 599.

## CHAPTER X. COMPLEMENT.

BY C. H. BROWNING (UNIVERSITY OF GLASGOW)

### Introduction.

IN Chapters VIII and IX it has been shown that the bactericidal and haemolytic actions of the sera of immunized animals, as well as other cytotoxic effects, are due to the combined action of an immune antibody along with a property of normal blood-serum or plasma, which is now generally known as complement. Also natural bactericidal and haemolytic action has been found in most cases to have a similar dual mechanism. As Bordet (1895) noted, the complementing property is not increased in fresh immune serum as compared with fresh normal serum, and this has also been clearly shown by Dungern (1900) and Bulloch (1901). In the first place the chief characters of complement already referred to will be summarized. Owing to the ease with which haemolytic effects are investigated, work on complement has been carried out mainly upon haemolysins. Ehrlich and Morgenroth (1899) made the following observations : (1) when complement-containing serum is treated with excess of red cells of a species for which the serum contains no haemolytic antibody and afterwards the cells are removed by centrifuging, the supernatant fluid is as rich in complement as before treatment ; (2) when red cells are treated with a mixture of immune-body (not in excess, see pp. 337, 339) and complement at a temperature of 0 to 3° C. haemolysis does not occur and the supernatant fluid obtained after centrifuging contains only the complement. Also the separated corpuscles when freed from serum and placed at 37° C. do not undergo lysis. The result contrasts with what occurs in mixtures kept at 37° C., since lysis takes place and complement is used up. Accordingly, complement has practically no affinity for red cells in the absence of immune-body ; and even in the presence of immune-body complement does not combine with the cells at low temperatures and hence does not exert haemolytic action. Two properties of complement may be distinguished, (1) combining action, and (2) toxic action. Wherever the latter is manifested combination has occurred, but combination may take place without any obvious toxic effects following. As Bordet (1900, 1909<sup>1</sup>) has pointed out, a serum can be deprived of all its complement action by appropriate treatment with sensitized red cells or bacteria.

### Definition and General Properties.

Complement—also known as alexin, addiment or cytase—is difficult to define completely ; Muir's (1909) definition of it as ' that labile substance of normal serum which is taken up by the combination of an antigen and its antisubstance (immune-body)' , is of very wide application. In order

to detect complement it is necessary to have a test object, commonly red corpuscles together with a suitable immune-body ; these sensitized red cells undergo haemolysis when complement is present, whereas unsensitized red cells remain intact. But the property of complementing the complex, red cells *plus* immune-body, so that lysis will result, depends in the case of serum of a given species on the capacity of the particular red cell *plus* the immune-body to bring the complement into combination. Even when this combination has been effected the complement may be so weakly toxic that lysis fails to occur. The action of complement may also be shown by bactericidal and bacteriolytic effects when suitable bacteria sensitized with corresponding immune-body are used, or by the solution of the precipitate obtained by the interaction of syphilitic serum with certain lipoidal substances (see Taniguchi, 1921). Complement may also act as opsonin ; this property is dealt with in a separate section. By means of appropriate combinations the presence of complement has been demonstrated generally in warm-blooded animals and it has also been shown to exist in the blood of certain lower vertebrates (see Mazzetti, 1913).

It is of importance that many antigens together with the corresponding antibodies, are able to remove the complementing property from sera *in vitro* (reaction of Bordet and Gengou, 1901). The complement is then said to have been absorbed or fixed. A good example of such a complement-fixing combination is a mixture in suitable proportions of serum with an antiserum obtained by injecting an animal of foreign species with the serum in question (Gengou, 1902). Since no obvious change may accompany the absorption, it is demonstrated by introducing into the mixture sensitized red cells susceptible to the complement used ; if these remain unlysed in the fluid, then it is evident that the complement has been removed.

A number of inorganic and organic substances, especially in the particulate or colloidal state, are capable of removing complement from fresh sera, e.g. among organic substances, cells of the various organs of the animal which furnishes the complement as well as of animals of different species, various bacteria and yeasts (Dungern, 1900 ; Friedberger and Putter, 1920), stromata of red corpuscles when used in sufficient amounts (Muir, 1903), also specific serum-antiserum precipitates. But it is the union of complement with cells brought about through the mediation of immune-body which is of special biological importance. With sensitized bacteria or red cells combination appears to be most active at 37° C., the effect of higher temperatures being complicated by the thermo-lability of complement. In other cases, however, fixation of complement may occur at lower temperatures. Increased concentration of sodium chloride or other electrolytes beyond the isotonic point hinders combination of complement with sensitized red corpuscles (see Nolf, 1900 ; Hektoen and Ruediger, 1904 ; Topley, 1915 ; Wright and MacCallum, 1922). Accordingly, the phenomena of combination are usually observed in isotonic

salt solution. Generally the combination is a firm one and dissociation occurs only to a minimal degree, if at all. The complements of different animal species may have the same combining mechanism in this respect, namely that the complement of one animal may keep out of combination that of another species.

The union of complement with an antigen and the corresponding antibody shows often in striking fashion the 'Ehrlich phenomenon'. That is to say, when the amount of complement is reached which is not completely absorbed, much more than a dose must be added further before one dose of complement remains free; the explanation of this is dealt with later (p. 343). The view has been advanced that the combination of complement is not essential for the occurrence of lysis, but is merely an accessory or 'post-haemolytic' phenomenon; but there is no satisfactory evidence in support of this (Bordet, 1911; Eagle and Brewer, 1929).

Complement produces its toxic action quantitatively, in the sense that the minimum amount of a specimen of fresh serum can be determined which will cause complete lysis of a given quantity of red corpuscles along with sufficient immune-body; this amount of complement is termed the minimum haemolytic dose (m.h.d. or 'dose'). In this respect the action of complement does not correspond with that of a ferment. When less than the optimum amount of immune-body is present more complement is required to effect lysis. With fractions of the m.h.d. of complement the amount of lysis which results is not proportional to the amount of complement present, thus a quarter of a dose may produce little or no lysis. Sometimes the toxic action of complement is most marked when before it is added a considerable time has been allowed for combination of the immune-body with the red corpuscles (Muir and Browning, 1906). In experiments involving the action of complement it is important to work with constant volumes, since the effect of complement depends greatly on its concentration as well as on the absolute amount present (Kiss, 1909; Scheller, 1910; Thiele and Embleton, 1914). Certain electrolytes, e.g. sodium or potassium acetate, appear to be more favourable to the action of complement than sodium chloride, so that in a medium of the former the lytic dose is lowered (Wright and MacCallum, 1922; Tokunaga, 1928). Other electrolytes, e.g. sodium citrate (Bordet and Gay, 1908; Gengou, 1908) and magnesium sulphate, inhibit the action of complement, even in isotonic solution (Tokunaga, 1928).

When serum is heated at a temperature short of that which produces coagulation of its proteins, e.g. at 55° C., the complementing action is, as a rule, destroyed. It may be shown, however, that although the toxic action has been removed (Ehrlich's toxophore or zymotoxic group), the combining property (haptophore group) may be still to a great extent retained; to this state the name 'complementoid' has been applied. Ehrlich and Sachs (1902) found that guinea-pig's corpuscles after treatment at 37° C. with heated dog's serum and subsequent washing, were insusceptible to the lytic action of fresh normal guinea-pig's serum, although

(1) lysis occurred when both sera were added simultaneously to the red cells and the mixture was then incubated ; again, (2) lysis occurred when the red cells were treated with heated dog's serum at 0° C. and after washing were brought into contact with guinea-pig's complement at 37° C. This result, which Ehrlich and Sachs attributed to the combination of complementoid preventing that of complement, is exceptional. Usually the existence of complementoid is demonstrated by taking advantage of the fact that after haemolysis has been produced by a small amount of complement and complementoid is then added, the latter is able to combine and so prevents further complement, which is added later, from being absorbed (Muir and Browning, 1904). As tested quantitatively in this way, the amount of complementoid which is formed may sometimes be equal to the complement originally present in the serum, but often it is less. Further evidence as to the existence of complementoid is afforded by observations on the dosage of complement in different media (Chapter IX).

### **Complement-fixation.**

It has been seen that complement may be powerfully absorbed by a mixture of antigen and antibody, although the anticomplementary effect of each constituent by itself may be negligible. This property is conferred in varying degree by antibodies to formed elements, such as bacteria, red corpuscles and tissue-cells, and also by those to proteins in solution (serum, milk, &c.), although it is usually very slight in the case of diphtheria antitoxin. Further, it must be noted that the complement-fixation brought about by a given antiserum does not necessarily run parallel in a quantitative sense with the other antibody manifestations which it may exhibit in co-operation with complement, e.g. bactericidal action. The reaction, however, has been found useful as a means of detecting antigens or antibodies. In order to examine a serum for the presence of a particular antibody one adds the corresponding antigen and also complement ; then, after allowing sufficient time for interaction, susceptible sensitized red cells are added and the mixtures are kept at 37° C. for a period. Sensitized bacteria may be used similarly as the indicator. If lysis or bactericidal action takes place this shows that the complement originally present has persisted in the active state—negative result ; on the other hand, if the cells or bacteria remain intact the complement has been fixed in the first stage of the reaction—positive result. A slight degree of the reaction may appear merely in a retardation of lysis of the test corpuscles. It is, of course, essential that the reaction be tested under quantitative conditions as regards the amounts of complement used. Further, in order that complement should be fixed there must be, in addition to a sufficiency of antigen, an optimum proportion of antibody to antigen, because excess of either may cause a zone of non-fixation. The results ought always to be controlled by determining the action of the serum and the antigen separately on

complement, since one or both of the reagents may have some anti-complementary action. It is worthy of note that some antigens, for instance certain bacteria (many strains of *B. typhosus*, &c.), may be anticomplementary when the complement is present in higher concentrations, but may have no action when it is more dilute (p. 341) : therefore it is essential to control the action of the antigen by itself with a wide range of doses of complement (Dunlop, 1928). By means of such controls one can ascertain that the inhibitory action of the mixture on complement is not due to either of the constituents alone. Also, in order to determine that the result is not merely a summation of effects, each separate constituent in the control tests is often used as a multiple, say double the quantity, of that present in the mixture. Valuable information on this point is yielded by a parallel test showing that complement-fixation fails to occur where normal serum of the same species is present instead of the antiserum. A positive result under controlled conditions affords evidence that the serum contains an antibody to the antigen employed. Similarly, an antigen which is suspected to be present can be identified by adding the corresponding antiserum and examining for complement-fixation. Many valuable practical applications of the method are dealt with under the various infections.

Weil and Felix (1920, 1921) concluded in the case of various bacteria of the *Proteus* and typhoid-paratyphoid groups that complement fixation is brought about principally by the heat-stable antigenic constituent (O antigen) together with the corresponding antibody, and not by the antibody which reacts with the thermo-labile (H) antigen. A similar result has been obtained by Felix and Robertson (1928) with anaerobes (*B. tetani* and *V. septique*). The evidence for this, according to the latter observers, is that (a) an antiserum fixes complement to the same degree with a bacillary antigen containing both the O and H constituents as with one containing the former alone ; (b) pure anti-O immune-sera are capable of leading to marked fixation of complement ; (c) the complement-fixing power of the antiserum is independent of its content in agglutinins for the H component of the organisms ; and (d) antisera which contain both O and H agglutinins are deprived of their power of fixing complement if the O agglutinins are removed by treatment with bacilli heated to 100° C., although under these conditions the H agglutinins of the serum are unchanged. Others, for instance Hofmeier (1927), working with *B. typhosus*, and Springut (1927) with the bacilli of mouse typhoid, have stated that the 'ectoplasmic' (H) antibodies (obtained, e.g. in the case of *B. typhosus*, after absorbing an antityphoid serum with a culture of this organism grown on phenol-containing agar) together with the corresponding (thermo-labile) antigenic components of the organisms also give rise to complement-fixation. White (1926) has found that the labile (H) antigenic components of members of the *Salmonella* group fix complement in the presence of antibody, but that the degree appears to be very small as compared with that due to the stable factors.

In the case of a precipitating antiserum complement-fixation occurs with amounts of the corresponding antigen much smaller than are necessary to produce obvious precipitation ; therefore the reaction is extremely delicate (see Muir and Martin, 1906<sup>1</sup>). Dean (1912<sup>1</sup>) has investigated the relation of the phenomena and has shown clearly that the optimum proportions between antigen and antibody are different in the two cases. The physical conditions modify the results markedly, complement being fixed more intensely when precipitation is in course of occurring than when it is complete ; that is, complement fixation is a function of the size of the particles of a precipitate (Goldsworthy, 1928). Therefore the order in which the reagents are mixed may be of importance.

Various other physical and chemical factors may affect the complement-fixation reaction. Differences in temperature may play an important part. Sensitized red corpuscles fix practically no complement at 0° C., provided that great excess of immune-body is not present, whereas at higher temperatures complement is fixed, the process being very active at 37° C. On the other hand, in mixtures of serum or bacterial extract with the corresponding antisera, while fixation of complement occurs more slowly at 0° C. than at 37° C., the amount eventually fixed is greater at the former temperature (Dean, 1917). The hydrogen-ion concentration may also influence the result ; thus, Browning and Wilson (1909) observed that a slightly acid reaction increased the amount of complement fixed by a mixture of globin and antiglobin serum. Kondo (1922) has drawn attention to the influence of the salt-content, diminution of which in certain cases favours fixation.

In some circumstances an antibody may fix complement when added to isolated constituents of the antigen, haptens. These, while essential constituents, may not by themselves act as antigens *in vivo* (p. 206). Haptens are often either of lipoidal nature (e.g. lecithin) or associated with lipins. The state of dispersion of the lipoidal emulsion has then a marked influence, more complement being fixed in the presence of a turbid emulsion than with a clear emulsion. Also, the presence of cholesterol intensifies the reactivity of the lipoidal emulsion ; this was first observed in the Wassermann reaction, but is also the case in complement-fixation due to Forssman's antibody along with the corresponding lipins (Chapter IX) ; the reason is obscure.

In the foregoing considerations complement has been treated as a whole, the final test for its presence being the occurrence of haemolytic or bactericidal action. As will be seen later, however, complement action depends on the co-operation of a series of fractions, the absence of any one of which may lead to its inactivation. When the behaviour of the different fractions is examined in complement-fixation reactions it is found that varying results are obtained (p. 343).

The further question arises as to whether substances which do not stand in the relationship of antigen to antibody, may when present

together cause complement-fixation although separately they lack anti-complementary action, as Noguchi (1910) found. This problem has been investigated particularly by Takenomata (1924) and by Mackie and Finkelstein (1928). The latter observed that mixtures of peptone and cholesterol were specially active in this respect. Frequently complement is fixed by such mixtures only when the serum is unheated; but in certain species, e.g. rabbit, ox, &c., the active principle of the serum resists heating at 55° C. Attempts have been made to distinguish 'genuine' complement-fixation due to antigen-antibody complexes from such 'pseudo-reactions' by varying the conditions under which the components interact; thus, according to Takenomata, specific antigen-antibody complement-fixation is marked at 0° C. as well as at 37° C., whereas pseudo-reactions occur only at the higher temperature. However, this is not a general rule, as the results with haemolytic antibodies show. At present, therefore, it is impossible to decide whether or not such results are due to a property of the serum which actually has the character of a natural antibody.

### Deviability.

By deviability is meant the capacity of complement for being fixed by an antigen-antibody complex. It has been seen that complementing comprises both combining and toxic actions and that these two functions do not necessarily run parallel in different complement-containing sera. The discrepancy, which may be met with in different specimens of serum from the same species, such as the guinea-pig, has considerable practical importance in the performance of complement-fixation tests, since when an antigen-antibody complex does not act intensely it may fail to be detected on using a complement whose combining property (deviability) is weak. The deviability of complement has been studied most extensively in connection with the Wassermann reaction (see Browning and Mackenzie, 1924). In general, deviability decreases more rapidly than haemolytic action when serum is kept in the fluid state; also, complement which is reconstituted after dialysis is less deviable than that of the same serum untreated (see Browning and Mackie, 1914, 1925). Treatment of complement with vegetable charcoal does not readily affect the deviability (Dunlop, 1928). On the other hand, deviability may be increased by shaking serum diluted with normal saline (A. Klopstock, 1924). The deviability of the complement of serum recently separated from defibrinated blood tends to be higher than after it has stood for some hours. Hence it is generally advantageous to use as the source of complement for fixation tests serum which has been kept overnight on ice, as the complement becomes more stable in consequence (see Browning and Mackenzie, 1924). Sera of different species may differ markedly in their deviability, as Muir and Martin (1906<sup>1</sup>) have shown for mixtures of serum with the corresponding antiserum.

### The 'Multiplicity' of Complements.

As has been seen, powerful absorbers, e.g. excess of sensitized bacteria, or red cells or stromata sensitized with the corresponding immune-body, may remove all the complement from a serum, and Bordet (1901, 1909<sup>2</sup>) therefore considered that complement is homogeneous in nature. But when milder absorbing reagents are employed it can be shown that the complement-content of serum is not uniform, since selective changes now result. Thus, Ehrlich and Sachs (1902) treated unheated normal goat's serum for a short time with rabbit's red corpuscles and then tested its complementing power for rabbit's and dog's corpuscles sensitized with the respective immune-bodies from the goat. The result showed that whereas the lytic dose of untreated serum was nearly the same for both systems, the dosage of the treated serum for sensitized dog's corpuscles was almost seven times as great as before, but for sensitized rabbit's corpuscles it was unaltered. Thus, the natural antibody for rabbit's corpuscles present in goat's serum led to a differential absorption of the complement. Again, the bactericidal action of normal serum for one species of organisms can be greatly reduced by treatment with small numbers of killed organisms of other species without the content of the serum in haemolytic complement being appreciably altered. Therefore, it appears that there is a portion of the complement which is mainly concerned in producing natural bactericidal action, *bacteriophilic complement* (Muir and Browning, 1908). However, restoration of the bactericidal action of the treated serum occurs on the addition of a specific immune-body for the organisms under test; in view of Dunlop's work (p. 345) the existence of a relatively non-specific, natural antibody might explain the results. In general, however, the evidence points to the presence in any serum of complements which show varying gradations in combining and toxic properties (Muir, 1909).

The behaviour of the separated 'fractions' of complement in fixation reactions will be dealt with later.

### Constitution of Complement especially in relation to Serum-Proteins.

When complement-containing serum of the guinea-pig is dialysed (Ferrata, 1907) or treated with a weak solution of hydrochloric acid (Sachs and Altmann, 1909) or with carbonic acid gas after adding water (Liefmann, 1909), so that a portion of the globulins separates out, it is found frequently (not invariably) that neither the fluid portion nor a solution of the precipitate in saline will lyse sensitized red corpuscles. But when the two constituents are mixed at once in the original proportions and the salt-content of the fluid is adjusted to normal the lytic action is restored (see Browning and Mackie, 1925). The portion which precipitates on dialysis corresponds to the part insoluble in acid—improperly named 'globulin fraction', since it contains the euglobulin and only a part of the pseudoglobulin. It has been called 'mid-piece' (Brand, 1907) in contrast to the 'end-piece' or soluble part ('albumin fraction'), because

red cells sensitized with immune-body fix the former in the isolated state, but not the latter. This fixation of mid-piece occurs at 0° C. (Hecker, 1907). Further, if red cells together with a great excess of immune-body are brought into contact with whole serum at 37° C. in a medium which has a suitable slightly acid reaction from the presence of acid sodium phosphate (Michaelis and Skwirsky, 1909), or at 0° C. in normal salt solution (Sachs and Bolkowska, 1910), the complement in the fluid may become inactivated owing to the removal of the mid-piece, which is fixed by the sensitized red cells without lysis occurring. The same result may follow when red cells along with excess of immune-body are brought into contact with complement in hypertonic salt solution at 37° C. (Guggenheim, 1911). Sensitized corpuscles which have fixed mid-piece are said to be 'persensitized', i.e. on the addition of end-piece they undergo lysis at 37° C. The mid-piece when kept in normal saline soon alters, so that on the addition of end-piece the complement activity is not restored (Brand's modification); but the modified mid-piece still retains its capacity to persensitize corpuscles in the presence of immune-body. The resistance of mid-piece and end-piece to heating is variable (see p. 344).

The autolysin of a paroxysmal haemoglobinuric's serum when added to the patient's own red cells has been shown to fix certain constituents of the homologous complement at 0° C.; the treated serum was not reactivated by the addition of mid-piece (Browning and Watson, 1912). Coca and Cooke (1914) found that similar treatment removed the end-piece of the complement so far as concerned its lytic action on sheep's corpuscles sensitized with immune-body from the rabbit, but did not affect the end-piece, which was effective in the lysis of rabbit's corpuscles along with the normal antibody present in the serum. The treatment removed most of the mid-piece as tested with both these haemolytic systems.

In many instances complement can be reconstituted by mixing end-piece from a certain species (guinea-pig) with mid-piece from another (rabbit, horse, ox, &c.); and rabbit's serum, for example, is as rich in mid-piece as guinea-pig's serum. On the other hand, rabbit's end-piece is not reactivated by either guinea-pig's or rabbit's mid-piece (Braun, 1911<sup>1</sup>; Browning and Mackie, 1925). As regards the quantitative relationships between mid-piece and end-piece of guinea-pig's serum, it is found that the end-piece derived from one dose of complement requires for its reactivation the mid-piece of not less than one dose and vice versa. Excess of end-piece, however, will cause lysis in the presence of a smaller quantity of mid-piece, but the reverse does not hold. The addition of end-piece to whole complement increases the haemolytic activity.

In addition to these two fractions of complement others have also been described. Thus, guinea-pig's complement which has been deprived of lytic action by treatment with cobra-venom ('venomed complement') is reactivated by the addition of heated guinea-pig's serum (54 to 55° C. for 15 to 30 minutes), although the latter has the properties neither of mid-piece nor end-piece (Ritz, 1912). This constituent has been called

'third component'; it is found mainly in the globulins obtained from serum by ammonium sulphate. Certain other agencies which inactivate complement appear to exert their effect also by removing third component, for example treatment with suspensions of *B. prodigiosus* at 37° C. (Ritz and Sächs, 1911, 1917); it is noteworthy that a given bacterial suspension may fail to remove the complement when the latter is dilute. Dunlop (1928) has made a similar observation in the case of most strains of *B. typhosus*, as well as with certain other organisms. The inactivation of complement by water appears to be due to action on third component (Hirschfeld and Klinger, 1915), and this is also the result of adding heparin *in vitro* (Ecker and Gross, 1929). According to Weil (1913), sensitized cells when treated with venom complement at 37° C. may fix mid-piece and end-piece; if they are then washed and resuspended in saline lysis occurs at 37° C. when third component is added, but the latter is stated not to be fixed by such 'hypersensitized' cells; also lysis does not occur in the mixture at 0° C. (Nathan, 1914<sup>1</sup>). Pig's serum is specially rich in third component (Jonas, 1913), and very minute amounts may suffice to reactivate complement (see Hyde, 1923). As regards the identity of third component, it is remarkable that complement inactivated by treatment with yeast, which also removes third component (see Whitehead, Gordon and Wormall, 1925), may sometimes be reactivated by venom complement according to Coca (1914). A race of guinea-pigs has been bred whose blood is practically devoid of haemolytic complement (Moore) owing to the absence of third component (Coca, see Hyde, 1923).

Gordon, Whitehead and Wormall (1926) have shown that haemolytic complement which has been inactivated by not too intense treatment with ammonia can be reactivated on adding serum treated with zymin, which is devoid of third component. Hence, they applied the term 'fourth component' to the constituent destroyed by ammonia. The latter is found mainly in the 'albumin fraction'; it is not dialysable and resists heating at 56° C. for half an hour. The ammonia does not interfere with the protein constituents or the phosphorus partition of the serum (Gordon and Marshall, 1929). Complement inactivated by treatment with ammonium sulphate is also reactivated by the addition of serum heated at 56° C.; the reactivating property is found to a certain extent both in the sediment and in the supernatant fluid when guinea-pig's serum is dialysed at a low temperature, 5° C. (Tokunaga, 1929<sup>1 & 2</sup>).

The above observations on the constitution of haemolytic complement have been confirmed in general for bactericidal complement of the guinea-pig. The latter may be split into inactive mid-piece and end-piece components, which, when mixed, reconstitute the complement (Braun, 1911<sup>2</sup>). Böhncke (1912) has also shown that venom complement is reactivated to a certain extent by the mid-piece fraction and less well by the end-piece. The action of ammonia causes a parallel loss of bactericidal power and haemolytic complement in normal guinea-pig's serum. Also, serum inactivated by ammonia has its bactericidal

action restored by the addition of heated serum (56° C. for half an hour), but not by serum which after heating has also been treated with ammonia (Gordon and Wormall, 1928<sup>2</sup>).

Browning and Mackie (1914, 1925) have investigated the effect on haemolytic complement of fractioning by means of ammonium sulphate whole serum or the constituents obtained after splitting with carbonic acid. In the case of guinea-pig's serum, there may be a distribution of the components of complement among four fractions—pseudo-globulin of mid-piece and of end-piece, euglobulin and albumin. Sometimes the complement can be practically reconstituted by mixing the two inactive pseudo-globulin moieties derived respectively from the mid-piece and end-piece; or again, the addition of the albumin to the pseudo-globulin of the carbonic acid precipitate may practically restore the haemolytic action to its original value. It is to be noted that the activity of such mixtures does not represent merely a summation of the effects of the separate constituents. Sera of other species may show a different distribution of complement components (Mackie, 1920), since in human and rabbit-serum the complement which acts with immune-body is concentrated in the globulins. When two criteria of complement are employed, namely haemolytic action (1) along with immune-body, and (2) along with cobra-venom, it is found that there is no constant relationship between the behaviour of the serum fractions in the two cases. Further, the results of Cruickshank and Mackie (1912, 1913) suggest that in addition to the complement-content of serum which can be measured by the usual methods, there exist constituents capable of being modified in such a way that they take part in complementing action. Thus, on rapidly adding a suitable alcoholic solution of egg-yolk lecithin to recently separated guinea-pig's or rabbit's serum, and then passing carbonic acid gas, the soluble portion (end-piece) so obtained was by itself as effective in complementing action on sensitized red cells as the original serum; but at the same time the separated mid-piece was equal in amount to the mid-piece obtained in the ordinary way. The end-piece fraction of the lecithin-serum mixture, therefore, contained mid-piece which could not be demonstrated as preformed in the original serum. Also, in the case of rabbit's serum the end-piece obtained after the addition of lecithin was much more active, as tested along with guinea-pig's mid-piece, than that obtained by the usual method. The addition of lecithin to separated mid-piece or end-piece produced no such enhancing effects.

According to Fuchs and his co-workers (see Fuchs, 1928, 1929<sup>1 & 2</sup>), the complement action of plasma is removed when the proserozyme is adsorbed by treatment with a suspension of magnesium hydroxide; restoration of the complement occurs on adding to the treated plasma the proserozyme extracted from the precipitate by the action of carbonic acid. Also it is concluded by them that prothrombin corresponds with mid-piece.

The general result is, therefore, that complement possesses great complexity of constitution which has not yet been completely elucidated.

Consequently, so-called fixation or absorption of complement may depend on the removal or masking of one or more of the various components, thus leading to all degrees of weakening of complementing action. This, no doubt, is in part the explanation of the Ehrlich phenomenon in haemolysis (p. 334), although here, as in bacteriolysis, both mid-piece and end-piece are stated to be absorbed (Skwirsky, 1910). But Browning and Mackie (1914, 1925) have found that complement absorbed with sensitized red corpuscles may be reactivated by end-piece alone, or by either end-piece or mid-piece, the former being the more effective of the two fractions. Where the complement is fixed and no obvious effects follow, e.g. after treatment with serum-antisera precipitates, it has been generally held that the mid-piece alone is removed; but according to Gengou (1911<sup>1 & 2</sup>) a portion of the end-piece is also fixed. Dean (1912<sup>2</sup>) has shown that the precipitate forming in a mixture of serum and anti-serum with complement at 0° C. fixes both mid-piece and end-piece; the mid-piece, although much the more readily fixed, can be recovered, but, on the other hand, this is not the case with the end-piece.

### Influence of Physical and Chemical Agents.

Complement is a very labile property of fresh serum. On keeping guinea-pig's serum, especially when diluted with saline (Ecker, 1922), for a few days at ordinary temperatures the complement becomes markedly weaker, and even within the first few hours after withdrawal of the blood and defibrinating a distinct fall may occur in the serum separated (see Morrison, 1922). F. Klopstock (1926) has found, however, that complement retains its properties better on standing if the plasma is obtained by mixing two volumes of blood with nine volumes of 0.3 per cent. sodium citrate in normal saline and then centrifuging. The complement of rabbit's serum, on the other hand, when kept on ice becomes more active in the course of several weeks (Pierret and Breton, 1925). Lumière and Grange (1928) conclude that the action of haemolytic complement depends on the content of the serum in carbonic acid, since arterial blood is less active than venous, and the latter becomes weaker when the serum is exposed for a short time to a partial vacuum. Bauer (1929) has found, however, that a 10 per cent. dilution of guinea-pig's complement in normal saline is inactivated on saturation with carbonic acid gas, but this effect is reversible. Light in the visible range of the spectrum is destructive particularly when fluorescent substances, e.g. eosin, are present in the fluid (Lichtwitz, 1904). Ultraviolet rays are still more harmful (see Lundberg, 1922; Friedberger and Scimone, 1923; Gordon and Wormall, 1928<sup>1</sup>). Exposure to  $\alpha$ -particles destroys complement (Chambers and Russ, 1911); but X-rays were stated by Scaffidi (1915) to be comparatively inactive in this respect, and although in the case of guinea-pigs Lusztig (1927) produced deterioration of the complement by irradiation either *in vivo* or *in vitro*, Andersen and Emmerich (1926) observed no immediate effects of such treatment *in vivo*.

Heating at 55 to 56° C. rapidly destroys complement action—in 5 or 6 minutes in the case of guinea-pig's serum (Schmidt, 1913); but more thermo-stable complements exist, e.g. that of certain goats' sera as tested with sheep's corpuscles resisted half an hour's heating (Ehrlich and Morgenroth, 1899). It is of interest that partial destruction of complement by brief heating may be followed by considerable restoration of activity when the serum is afterwards kept at 37° C. for several hours (see Brooks, 1920<sup>2</sup>). According to Coulter (1921) the effect of heating complement diluted with water depends on the pH, which influences the interaction of a euglobulin compound with other substances in the serum. When heat-inactivated serum is dialysed and the precipitate is then added to the soluble portion it occasionally happens that the mixture possesses complementing power (Mutermilch, 1911). The susceptibility of the mid-piece and end-piece fractions to heating has shown great variability according to different observers (see Schmidt, 1913; Wormall, Whitehead and Gordon, 1925), and when two criteria of complement action—activation of haemolytic immune-body and of cobra-venom haemolysin—are applied in parallel the results are still more anomalous (Browning and Mackie, 1914, 1925).

Caustic soda in appropriate concentrations inactivates complement, restoration occurring on neutralization; greater concentrations are destructive (see Hecker, 1907). Other more or less destructive agents are hydrochloric acid (see Hecker, 1907), proteolytic ferments—e.g. papain (Ehrlich and Sachs, 1902), pancreatic extracts (Michaelis and Skwirsky, 1910; Wormall, Whitehead and Gordon, 1925)—cobra-venom (see Browning and Mackie, 1914, 1925), shaking with ether (Kyes and Sachs, 1903) or chloroform (see Fraenkel, 1914), and the addition of sufficient proportions of alcohol and many other organic substances, e.g. alcoholic tissue extracts (see Browning and Mackenzie, 1924), bile-salts, soaps, also poisonous alkaloids (Goldschmidt and Pribram, 1909). It is of interest that many of these agents are also haemolytic and that the addition of serum inhibits both this and the anticomplementary action.

Great importance from the theoretical aspect has been attached by Sachs and his co-workers to the destruction of complement following agitation, even in the absence of air, noted by Jakoby and Schütze (see Schmidt, 1913, 1914), or following reduction of the salt-content brought about under suitable conditions by appropriate dilution with distilled water (Sachs and Teruuchi, 1907; Guggenheimer, 1910; Sachs and Altmann, 1917). Such procedures are held to affect complement by altering the labile globulins of the serum.

In addition to various organic materials—living and dead bacteria and animal cells—as already mentioned (p. 333), many substances in the particulate or colloidal state have the property of removing complement action, e.g. casein, cholesterol, kaolin (Landsteiner and Stankovic, 1906), shellac (Seligmann, 1907), treatment with kieselguhr or filtration through a Berkefeld filter (see Muir and Browning, 1909; Schmidt, 1914). According

to Browning and Mackie (1925) neither mid-piece nor end-piece restores the action of filtered guinea-pig's complement. Doubtless the physico-chemical process of adsorption accounts for the fixation in most of these instances. But with certain bacteria, e.g. *B. typhosus*, along with guinea-pig's serum, a natural antibody may be responsible for the fixation of complement, as Dunlop (1928) has suggested, since it is possible to sensitize the organisms by treatment with the serum at low temperatures, while the complement of the treated serum is not fixed on exposure to fresh organisms. Non-specific agents may remove this property ; thus, after treatment of the serum with powdered charcoal the complement is no longer fixed by the organisms, although its haemolytic activity and deviability in the Wassermann reaction may be unchanged. In the case of filtration through a Berkefeld filter absorption of the complement is prevented by the addition to the serum of 10 per cent. sodium chloride (Muir and Browning, 1909), which inhibits complement haemolysis ; after filtration the complement action can be restored by diluting till the salt-content is normal. Dyes, both basic (electro-positive, e.g. night-blue) and acid (electro-negative, e.g. congo-red), when dissolved in salt solution may inhibit complement (F. Klopstock, 1924). The addition of a lipoidal extract (Wassermann antigen) intensifies this action ; but the effect is diminished by adding serum to a solution of the dye or by mixing solutions of basic and acid dyes in suitable proportions. Gordon (1930) has shown that on treating an inactive mixture of fresh guinea-pig's serum and congo-red with charcoal, which removes the dye, the haemolytic complement is restored.

The existence of immune anticomplement in the sense of a true antibody to complement cannot be demonstrated, since, as Gengou and later Moreschi showed, the serum-proteins themselves act as antigens and give rise to complement-fixing antibodies (see Muir, 1909, also Chapter XIII). Normal sera, either unheated or especially after heating, e.g. at 50 to 56° C., are often markedly inhibitory to complement (see Surface and Routt, 1913). That the complement is masked and not destroyed has been shown in certain cases, e.g. the inhibition of guinea-pig's complement by heated guinea-pig's serum, since on diluting an inactive mixture with saline the complement is enabled to cause haemolysis. The anti-complementary action of heated serum appears, in part at least, to be due to the presence of derivatives of complement (see Chapter IX). Human serum which has been preserved under sterile conditions becomes intensely inhibitory toward guinea-pig's complement (see Browning and Mackenzie, 1924) ; this action is removed by brief heating at 56° C.

The important influence, which may be exerted by the physical state of the agent with which the serum is treated, is well exemplified by the observation of Sachs and Nathan (1914), that complement was removed by treating serum with a suspension of inulin, but not by a solution of that substance. Again, the degree of dilution may markedly affect the results, e.g. according to Takenomata (1924) the third component of

guinea-pig's serum is more resistant to heating at 55° C. for 30 minutes when the serum is diluted 1 in 10 with normal salt solution than when undiluted.

The term 'anticomplementary' is applied to those agencies which suppress the action of complement; often no distinction is made between reversible inhibition—such as that due to a hypertonic medium—and irreversible inactivation. In general, it may be said that procedures which tend to denature serum-proteins also inactivate complement irreversibly, but it is not possible to relate closely any physical alteration, such as change in surface tension, with loss of complementing power (see Schmidt, 1913, 1914; Brooks, 1920<sup>1</sup>), or change in reaction (Seligmann, 1908; Davidsohn, 1910; Hirsch and Peters, 1922).

Complement may be preserved indefinitely, as Morgenroth pointed out, by keeping the serum frozen, e.g. at -15 to -20° C.—the best method. It may be kept for a time by rendering the fluid hypertonic, e.g. by adding 4·25 per cent. NaCl and diluting to 1 in 5 before use, as suggested by Friedberger (1908), or by adding 25 per cent. glycerin. Rapid drying, according to the method of Hartley, Eagleton and Okell, is also effective (see Browning and Mackenzie, 1924). Storage of guinea-pig's serum in an atmosphere of carbon dioxide has a very marked preservative effect on the complement (Valley and McAlpine, 1928).

### **Functions, Mode of Action, Origin and Nature of Complement.**

#### *Functions.*

Reference has already been made to the part played by complement in bringing about bacteriolytic and bactericidal effects when acting on susceptible organisms which have been sensitized by the corresponding immune-body. These are exemplified in Pfeiffer's reaction, and there can be little doubt that this is one of the chief mechanisms which contribute to acquired antimicrobic immunity. The lysis of sensitized red blood corpuscles and other animal cells by cytotoxic serum has been generally accepted as a similar phenomenon and it has been held that the diffusion of haemoglobin out of the corpuscles is evidence of damage to the cells, since they lose their osmotic properties in the process (Bordet, 1900). In the case of natural lytic action exhibited by fresh serum, complement is also as a rule an essential factor. Further, complement contributes to the intracellular destruction of organisms by rendering them susceptible to phagocytosis, the opsonic action of normal serum depending on the presence of complement (Muir and Martin, 1906<sup>2</sup>), although the destruction of the fourth component does not affect this property (Gordon, Whitehead and Wormall, 1929). In addition, under special circumstances opsonic action, which is not of the nature of summation of effects, may be due to a combined action of complement and immune-body (Browning, 1908). In those instances in which complement becomes fixed through the mediation of immune-body or otherwise,

but no bactericidal or opsonic effect or other physical change follows, it is not known whether the phenomenon conduces to the production of immunity. In considering the functions of complement, especially as contributing to immunity, it must be remembered that the complement in any one individual is not homogeneous, and that natural bactericidal action is probably due to only a small portion of the complement which can be measured by haemolytic tests.

In addition, complement is responsible for the action of serum on red corpuscles along with colloidal silicic acid or with cobra-venom; but in the latter case the complement is not identical with that which acts with immune-body (see Browning and Mackie, 1913, 1925). The agglutinating action of ox's serum for some species of sensitized red cells, e.g. ox corpuscles sensitized with immune-body from the rabbit (Muir and Browning, 1906; Bordet and Gay, 1906), which occurs in the presence of complement, is probably not due to the complement itself, but to a constituent of the ox-serum so far unidentified—Bordet and Streng's 'conglutinin'. This was shown by Yoshinare (1922), who found that fractional filtration of ox's serum yielded certain filtrates which caused conglutination but lacked haemolytic complement.

#### *Mode of Action.*

As to the mode of action of complement there is no definite knowledge. It was originally supposed by Ehrlich that the mechanism of complement action was analogous to that by which living protoplasm subserved its nutrition. When complement was brought into combination with a susceptible cell through the medium of immune-body then the zymotoxic group came into operation and a sort of digestion was supposed to follow. According to such a conception complement was analogous to the ferments. But complement is not identical with serum protease, as is shown by the different effects of inactivating agents on the two (see Jobling, Eggstein and Petersen, 1915). Failure has also, as a rule, attended attempts to demonstrate proteolytic products resulting from the action of complement and antibody on the corresponding antigen, e.g. by the method of the Abderhalden reaction for red corpuscles (McVey, 1918), or by estimation of amino-nitrogen for proteins (McNeil and Kahn, 1918). Further, neither in the course of complement-fixation by bacteria, e.g. *B. pseudodysenteriae* along with the homologous antibody, nor in the Wassermann reaction were any changes detectable by the interferometer (see Bachmann, 1923, 1924). Accordingly, there is no evidence of the occurrence of processes of decomposition or synthesis such as are brought about by the recognized ferments. Neither is there convincing evidence that complement acts by effecting lipolysis (see Noguchi, 1907; Jobling and Bull, 1913; Olsen, 1921; Brinkman and Szent-Györgyi, 1924). Caution must, however, be exercised in deciding what is evidence of complement action; thus, the lytic effect of fresh guinea-pig's serum on unsensitized red corpuscles suspended in isotonic sugar solution has been attributed to direct action

of complement (see Topley, 1915 ; Georgi, 1920 ; Wright and MacCallum, 1922), since heated serum is without effect. But it must be noted that lysis may be produced by serum where complement action can be definitely excluded, e.g. lysis due to brilliant green and serum, which is manifested even when the serum has previously been boiled (Browning and Mackie, 1914, 1925). According to Brown and Broom (1929), the negative electric charge which complement bears, is important in connection with its action.

### *Origin.*

The origin of complement is obscure. Complement, both bactericidal and haemolytic, is practically equal in strength in blood-plasma and in serum, as Hewlett (1903) showed for goose's plasma, which is normally uncoagulable, and also for oxalate-plasma from the dog (see Addis, 1912), although this has been denied (see Fuchs, 1929<sup>2</sup>). Evidence as to the existence of free complement in the circulating plasma is afforded by the rapid occurrence of lysæmia when an animal is injected intravenously with an immune-body for its own red corpuscles or with some of its own red cells which have been sensitized with the corresponding immune-body *in vitro* (Muir and Mcnee, 1912<sup>1 & 2</sup>). Although the serum of normal mice lacks haemolytic complement, but has mid-piece action (Ritz, 1911) *in vitro*, haemolysis occurs *in vivo* after the injection of immune-body (Bieling and Isaac, 1922). Complement is also present in varying strength in lymph from exudates and transudates (see Marshall, 1905). Its presence in other body fluids would appear to be due to admixture with the above, e.g. the occurrence of complement in the aqueous humour as the result of paracentesis (see Schneider, 1908), and in the cerebrospinal fluid in meningitis (Kafka, 1912), although normal cerebrospinal fluid contains mid-piece which is thermo-labile (Kafka, 1923). In milk there is no complement (Bulloch, 1902) or only traces (see Lane-Claypon, 1908 ; Kolff and Noeggerath, 1909). Experiments conducted *in vivo* with a view to comparing the relation of complement to cellular constituents of the blood, e.g. by investigating the effects of haemorrhage or of the leucotoxic action of injections of benzene, have shown that there is no fixed relationship and therefore do not afford evidence that complement is formed by leucocytes or other blood-cells. In the former case in the guinea-pig, complement was rapidly regenerated and, in the latter in rabbits, complement was little affected (see Ecker with Rees, Kline and De Caluwe, 1922). Previous experiments of Lippmann and Plesch (1913) in guinea-pigs rendered leucopenic by treatment with thorium-X had similar results. Also, exposure of living guinea-pigs to X-rays has produced no constant effects. Earlier attempts to extract complement from leucocytes ground at the temperature of liquid air yielded negative results ; although, as tested by natural bactericidal action serum-complement was not damaged at -180° C. (Petrie, 1903). A participation of the reticulo-endothelial system in the formation of complement is suggested by the work of Jungeblut and Berlot (1926), who found that attempted 'blockade' of the

system produced by an intravenous injection of Indian ink in the guinea-pig was followed by a progressive fall in the complement-content of the blood for three hours; however, restoration to normal was complete within twenty-four hours. The observation of Ehrlich and Morgenroth (1900) that in rabbits poisoned with phosphorus complement was absent from the serum, has suggested that the liver takes part in the formation of complement. Dick's (1913) work with such liver poisons as chloroform and hydrazine supports this; whereas acute damage to the kidneys following administration of mercuric chloride did not affect the complement. Also, excision of various other organs had usually no influence on the complement. Again, prolonged perfusion experiments with sensitized sheep corpuscles (see Olsen, 1922) indicate that the guinea-pig's liver—which has been previously perfused till free from serum—secretes complement, which is, however, deficient in third component, since lysis of the corpuscles is hastened by adding heated guinea-pig's serum to the circulating suspension. But neither mid-piece nor end-piece can be extracted by macerating liver-pulp. In guinea-pigs the state of nutrition has no definite relation to the content of the serum in haemolytic complement, but a deficiency is associated with pregnancy (Griffith and Scott, 1920). In the human subject in many acute infective diseases, when of moderate severity, the haemolytic complement in the blood is frequently increased and this may also be the finding in fatal cases (Gunn, 1914). In infancy the complement content of the blood tends to be lowest shortly after birth and to undergo marked increase during the first three months of life (Findlay, Fua and Noeggerath, 1909). Langer and Kirklund (1921) have found in infancy a diminution of bactericidal complement where there was severe nutritional disturbance, whereas haemolytic complement was not notably affected. After anaphylactic shock a fall in complement has been frequently observed (see Friedberger and Hartoch, 1909), which may be simply an associated phenomenon. In general, the changes in disease have thrown little light on the question of the origin or function of complement (see Browning, 1913). Complement does not undergo marked or permanent increase as the result of immunization procedures. On the other hand, after a reduction has been produced *in vivo* restoration of the complement-content of the blood soon occurs.

#### *Nature.*

As regards the nature of complement, whether lipins are essential for its action is uncertain. The work of Cruickshank and Mackie (p. 342) is highly suggestive in this direction; on the other hand, it has been held (Liefmann and Cohn, 1910) that there is no evidence that lipins are a constituent of complement, and Dean (1913) found that the lipoidal substances extracted from complement-containing serum by treatment with alcohol and ether at 0° C. had not the properties either of mid-piece or end-piece. Attempts to prepare artificial complement, e.g. by mixtures of soap and serum (Noguchi, 1907; Liebermann and Fennyvessy, 1912;

Born, 1925), have not led to products resembling closely natural complement. It has already been seen that the complement function is bound up with the protein constituents of the blood fluid and that it is often distributed over several of the protein fractions, which act together and not merely after the manner of summation. Sachs and his co-workers attribute complement action, at least so far as concerns mid-piece, to 'lability of the globulins'; this theory is based on the conception that there is an essential similarity between the conditions which lead to lytic action of complement and to its inactivation (see Nathan, 1917; Sachs and Klopstock, 1928). At present, it is impossible to determine whether complement is of a compound nature, as is believed to be the case for enzymes, or whether it may represent a physico-chemical state of a mixture of proteins, lipins and other constituents of the serum (see Hecht, 1923).

## REFERENCES.

ADDIS, T., 1912, *J. Infect. Dis.*, **10**, 200.  
 ANDERSEN, E. & EMMERICH, E., 1926, *Z. ImmunForsch.*, **47**, 565.  
 BACHMANN, W., 1923, *Z. ImmunForsch.*, Tl. I, Orig., **35**, 462; 1924, *ibid.*, **40**, 325.  
 BAUER, R., 1929, *Z. ImmunForsch.*, **61**, 309.  
 BIELING, R. & ISAAC, S., 1922, *Z. ges. exp. Med.*, **26**, 251.  
 BOEHNCKE, K. E., 1912, *Z. ImmunForsch.*, Tl. I, Orig., **13**, 240.  
 BORDET, J., 1895, see BORDET, 1909<sup>1</sup>; 1900, *Ann. Inst. Pasteur*, **14**, 257; 1901,  
*ibid.*, **15**, 303; 1909<sup>1</sup>, *Studies in Immunity*, New York and London, pp. 8, 56;  
 1909<sup>2</sup>, *Z. ImmunForsch.*, Tl. II, Ref., **1**, 1; 1911, *ibid.*, Tl. I, Orig., **12**, 601.  
 BORDET, J. & GAY, F. P., 1906, *Ann. Inst. Pasteur*, **20**, 467; 1908, *ibid.*, **22**, 625.  
 BORDET, J. & GENGOU, O., 1901, *Ann. Inst. Pasteur*, **15**, 290.  
 BORN, J., 1925, *Z. ImmunForsch.*, **44**, 33.  
 BRAND, E., 1907, *Berl. klin. Wschr.*, **44**, 1075.  
 BRAUN, H., 1911<sup>1</sup>, *Biochem. Z.*, **81**, 65; 1911<sup>2</sup>, *Z. ImmunForsch.*, Tl. I, Orig., **9**, 665.  
 BRINKMAN, R. & SZENT-GYÖRGYI, A. V., 1924, *Biochem. Z.*, **146**, 212.  
 BROOKS, S. C., 1920<sup>1</sup>, *J. Gen. Physiol.*, **3**, 169, 185; 1920<sup>2</sup>, *J. Med. Res.*, **41**, 411.  
 BROWN, H. C. & BROOM, J. C., 1929, *Brit. J. Exp. Path.*, **10**, 387.  
 BROWNING, C. H., 1908, *J. Med. Res.*, **19**, 201; 1913, *Quart. J. Med.*, **6**, 399; 1918,  
*Applied Bacteriology*, London, p. 158; 1925, *Immunochemical Studies*, London,  
 p. 19.  
 BROWNING, C. H. & MACKENZIE, I., 1924, *Recent Methods in the Diagnosis and  
 Treatment of Syphilis*, London.  
 BROWNING, C. H. & MACKIE, T. J., 1913, *Z. ImmunForsch.*, Tl. I, Orig., **17**, 1;  
 1914, *ibid.*, Tl. I, Orig., **21**, 422; 1925, see BROWNING, C. H., 1925.  
 BROWNING, C. H. & WATSON, H. F., 1912, *J. Path. Bact.*, **17**, 117.  
 BROWNING, C. H. & WILSON, G. H., 1909, *J. Path. Bact.*, **14**, 174; see also BROWNING,  
 C. H., 1925.  
 BULLOCH, W., 1901, *Zbl. Bakt.*, Abt. I, **29**, 724; 1902, *Trans. Path. Soc.*, Lond.,  
**53**, 189.  
 CHAMBERS, H. & RUSS, S., 1911, *Proc. Roy. Soc.*, B, **84**, 124.  
 COCA, A. F., 1914, *Z. ImmunForsch.*, Tl. I, Orig., **21**, 604.  
 COCA, A. F. & COOKE, R. A., 1914, *Z. ImmunForsch.*, Tl. I, Orig., **21**, 632.  
 COULTER, C., 1921, *J. Gen. Physiol.*, **3**, 771.  
 CRUICKSHANK, J. & MACKIE, T. J., 1912, *Biochem. Z.*, **42**, 414; 1913, *J. Path. Bact.*,  
**18**, 99.  
 DAVIDSOHN, H., 1910, *Z. ImmunForsch.*, Tl. I, Orig., **5**, 182.  
 DEAN, H. R., 1912<sup>1</sup>, *Z. ImmunForsch.*, Tl. I, Orig., **13**, 84; 1912<sup>2</sup>, *J. Hyg., Camb.*,  
**12**, 259; 1913, *J. Path. Bact.*, **18**, 118; 1917, *ibid.*, **21**, 193.  
 DICK, G. F., 1913, *J. Infect. Dis.*, **12**, 111.  
 DUNGERN, E. v., 1900, *Münch. med. Wschr.*, **47**, 677.  
 DUNLOP, E. M., 1928, *J. Path. Bact.*, **31**, 769.

EAGLE, H. & BREWER, G., 1929, *J. Gen. Physiol.*, **12**, 845.  
 ECKER, E. E., 1922, *J. Infect. Dis.*, **31**, 356.  
 ECKER, E. E. & GROSS, P., 1929, *J. Infect. Dis.*, **44**, 250.  
 ECKER, E. E., KLINE, B. S. & DE CALUWE, 1922, *J. Infect. Dis.*, **31**, 368.  
 ECKER, E. E. & REES, H. M., 1922, *J. Infect. Dis.*, **31**, 361.  
 EHRLICH, P. & MORGENTHOTH, J., 1899, *Berl. klin. Wschr.*, **38**, 1, 481; 1900, *ibid.*, **37**, 453, 681.  
 EHRLICH, P. & SACHS, H., 1902, *Berl. klin. Wschr.*, **39**, 297, 335, 492.  
 FELIX, A. & ROBERTSON, M., 1928, *Brit. J. Exp. Path.*, **9**, 6.  
 FERRATA, A., 1907, *Berl. klin. Wschr.*, **44**, 366.  
 FINDLAY, L., FUJII, R. & NOEGGERATH, C. T., 1909, *Jahrb. Kinderhk.*, **70**, 732.  
 FRAENKEL, E., 1914, *Z. ImmunForsch.*, Tl. I, Orig., **20**, 299.  
 FRIEDBERGER, E., 1908, *Zbl. Bakt.*, Abt. I, Orig., **46**, 441.  
 FRIEDBERGER, E. & HARTOCH, O., 1909, *Z. ImmunForsch.*, Tl. I, Orig., **3**, 581.  
 FRIEDBERGER, E. & PUTTER, E., 1920, *Z. ImmunForsch.*, Tl. I, Orig., **30**, 227.  
 FRIEDBERGER, E. & SCIMONE, V., 1923, *Z. ImmunForsch.*, Tl. I, Orig., **37**, 341.  
 FUCHS, H. J., 1928, *Z. ImmunForsch.*, **58**, 14; 1929<sup>1</sup>, *ibid.*, **61**, 342; 1929<sup>2</sup>, *ibid.*, **62**, 107, 117.  
 GENGOU, O., 1902, *Ann. Inst. Pasteur*, **16**, 734; 1908, see BORDET, 1909, *Studies in Immunity*, New York and London; 1911<sup>1</sup>, *Z. ImmunForsch.*, Tl. I, Orig., **9**, 344; 1911<sup>2</sup>, *ibid.*, Tl. I, Orig., **11**, 143.  
 GEORGI, W., 1920, *Z. ImmunForsch.*, Tl. I, Orig., **29**, 92.  
 GOLDSCHMIDT, R. & PRIBRAM, E., 1909, *Z. exp. Path. Ther.*, **6**, 211.  
 GOLDSWORTHY, N. E., 1928, *J. Path. Bact.*, **31**, 220.  
 GORDON, J., 1930, *J. Path. Bact.*, **33**, 47, 689.  
 GORDON, J. & MARSHALL, P. G., 1929, *Brit. J. Exp. Path.*, **10**, 249.  
 GORDON, J., WHITEHEAD, H. R. & WORMALL, A., 1926, *Biochem. J.*, **20**, 1028, 1036; 1929, *J. Path. Bact.*, **32**, 57.  
 GORDON, J. & WORMALL, A., 1928<sup>1</sup>, *Biochem. J.*, **22**, 909; 1928<sup>2</sup>, *J. Path. Bact.*, **31**, 753.  
 GRIFFITH, F. & SCOTT, W. M., 1920, *Rep. Publ. Hlth. Med. Subj.*, No. 1, Lond.  
 GUGGENHEIMER, H., 1910, *Z. ImmunForsch.*, Tl. I, Orig., **8**, 295; 1911, *ibid.*, Tl. I, Orig., **11**, 393.  
 GUNN, W. C., 1914, *J. Path. Bact.*, **19**, 155.  
 HECHT, H., 1923, *Z. ImmunForsch.*, Tl. I, Orig., **36**, 321.  
 HECKER, R., 1907, *Arb. Inst. exp. Ther. Frankfurt, Hft.* **3**, 39.  
 HEKTOEN, L. & RUEDIGER, G. F., 1904, *J. Infect. Dis.*, **1**, 379.  
 HEWLETT, A. W., 1903, *Arch. exp. Path. Pharmak.*, **49**, 307.  
 HIRSCH, E. F. & PETERS, E. C., 1922, *J. Infect. Dis.*, **30**, 263.  
 HIRSCHFELD, L. & KLINGER, R., 1915, *Biochem. Z.*, **70**, 398.  
 HOFMEIER, K., 1927, *Z. ImmunForsch.*, **50**, 71.  
 HYDE, R. H., 1923, *J. Immunol.*, **8**, 267.  
 JOBLING, J. W. & BULL, C. G., 1913, *J. Exp. Med.*, **17**, 61.  
 JOBLING, J. W., EGGSTEIN, A. A. & PETERSEN, W., 1915, *J. Exp. Med.*, **21**, 239.  
 JONAS, W., 1913, *Z. ImmunForsch.*, Tl. I, Orig., **17**, 539.  
 JUNGEBLUT, C. W. & BERLOT, J. A., 1926, *J. Exp. Med.*, **43**, 797.  
 KAFKA, V., 1912, *Z. ges. Neurol. Psychiat.*, Orig., **9**, 132; 1923, *Z. ImmunForsch.*, Tl. I, Orig., **37**, 315.  
 KISS, J., 1909, *Z. ImmunForsch.*, Tl. I, Orig., **3**, 558.  
 KLOPSTOCK, A., 1924, *Z. ImmunForsch.*, **41**, 126.  
 KLOPSTOCK, F., 1924, *Biochem. Z.*, **149**, 331; 1926, *Zbl. Bakt.*, Abt. I, Orig., **98**, 100.  
 KOLFF, W. & NOEGGERATH C. T., 1909, *Jahrb. Kinderhk.*, **70**, 701.  
 KONDO, S., 1922, *Z. ImmunForsch.*, Tl. I, Orig., **35**, 366.  
 KYES, P. & SACHS, H., 1903, *Berl. klin. Wschr.*, **40**, 21, 57, 82.  
 LANDSTEINER, K. & STANKOVIC, R., 1906, *Zbl. Bakt.*, Abt. I, Orig., **42**, 353.  
 LANE-CLAYPON, J. E., 1908, *J. Path. Bact.*, **18**, 34.  
 LANGER, H. & KIRKLUND, R., 1921, *Z. Kinderhk.*, **27**, 302.  
 LICHTWITZ, L., 1904, *Münch. med. Wschr.*, **51**, 1589.  
 LIEBERMANN, L. & FENYVESSY, B., 1912, *Z. ImmunForsch.*, Tl. I, Orig., **13**, 695.  
 LIEFMANN, H., 1909, *Münch. med. Wschr.*, **56**, 2097.  
 LIEFMANN, H. & COHN, M., 1910, *Z. ImmunForsch.*, Tl. I, Orig., **6**, 88.

LIPPMANN & PLESCH, 1913, *Z. ImmunForsch.*, Tl. I, Orig., **17**, 548.  
 LUMIÈRE, A. & GRANGE, 1928, *C.R. Acad. Sci.*, Paris, **186**, 1654.  
 LUNDBERG, E. G., 1922, *J. Immunol.*, **7**, 389.  
 LUSZTIG, A., 1927, *Zbl. Bakt.*, Abt. I, Orig., **105**, 142.  
 MACKIE, T. J., 1920, *J. Immunol.*, **5**, 379; see also BROWNING, C. H., 1925, *Immunochemical Studies*, London.  
 MACKIE, T. J. & FINKELSTEIN, M. H., 1928, *J. Hyg., Camb.*, **28**, 172.  
 MCNEIL, A. & KAHN, R. L., 1918, *J. Immunol.*, **3**, 295.  
 MCVEY, 1918, see BROWNING, 1918.  
 MARSHALL, H. T., 1905, *J. Exp. Med.*, **6**, 347.  
 MAZZETTI, L., 1913, *Z. ImmunForsch.*, Tl. I, Orig., **18**, 132.  
 MICHAELIS, L. & SKWIRSKY, P., 1909, *Z. ImmunForsch.*, Tl. I, Orig., **4**, 357, 629; 1910, *ibid.*, Tl. I, Orig., **7**, 497.  
 MORRISON, L. F., 1922, *J. Immunol.*, **7**, 435.  
 MUIR, R., 1903, *Lancet*, Lond., ii, 446; 1909, *Studies on Immunity*, London.  
 MUIR, R. & BROWNING, C. H., 1904, *Proc. Roy. Soc.*, **74**, i, 298; 1906, *J. Hyg., Camb.*, **6**, i, 20; 1908, *J. Path. Bact.*, **13**, 76; 1909, *ibid.*, **13**, 232.  
 MUIR, R. & MCNEE, J. W., 1912<sup>1</sup>, *J. Path. Bact.*, **16**, 410; 1912<sup>2</sup>, *ibid.*, **17**, 92.  
 MUIR, R. & MARTIN, W. B. M., 1906<sup>1</sup>, *J. Hyg., Camb.*, **6**, 265; 1906<sup>2</sup>, *Brit. Med. J.*, ii, 1783.  
 MUTERMILCH, S., 1911, *C.R. Soc. Biol.*, Paris, **70**, 577.  
 NATHAN, E., 1914<sup>1</sup>, *Z. ImmunForsch.*, Tl. I, Orig., **21**, 259; 1914<sup>2</sup>, *ibid.*, **23**, 204; 1917, *ibid.*, Tl. I, Orig., **28**, 503.  
 NOGUCHI, H., 1907, *Biochem. Z.*, **8**, 185, 327; 1910, *Proc. Soc. Exp. Biol.*, N.Y., **7**, 55.  
 NOFL, P., 1900, *Ann. Inst. Pasteur*, **14**, 656.  
 OLSEN, O., 1921, *Biochem. Z.*, **124**, 119; 1922, *ibid.*, **133**, 24.  
 PETRIE, G. F., 1903, *J. Path. Bact.*, **9**, 130.  
 PIERRET & BRETON, 1925, *C.R. Soc. Biol.*, Paris, **93**, 1612.  
 RITZ, H., 1911, *Z. ImmunForsch.*, Tl. I, Orig., **9**, 321; 1912, *ibid.*, **13**, 62.  
 RITZ, H. & SACHS, H., 1911, *Zbl. Bakt.*, Abt. I, Ref., **50**, 43\*; 1917, *Z. ImmunForsch.*, Tl. I, Orig., **26**, 483.  
 SACHS, H. & ALTMANN, K., 1909, see SACHS in *Handbuch der Technik u. Methodik der Immunitätsforschung*, hrsg. R. Kraus u. C. Levaditi, Jena, **2**, 895; 1917, *Biochem. Z.*, **78**, 46.  
 SACHS, H. & BOLKOWSKA, G., 1910, *Z. ImmunForsch.*, Tl. I, Orig., **7**, 778.  
 SACHS, H. & KLOPSTOCK, A., 1928, *Handbuch der biologischen Arbeitsmethoden*, hrsg. E. Abderhalden, Abt. XIII, Teil 2 (Lfg. 250), Berlin.  
 SACHS, H. & NATHAN, E., 1914, see NATHAN, E., 1914<sup>2</sup>.  
 SACHS, H. & TERUUCHI, Y., 1907, *Berl. klin. Wschr.*, **44**, 467, 520, 602.  
 SCAFFIDI, V., 1915, *Biochem. Z.*, **69**, 162.  
 SCHELLER, R., 1910, *Zbl. Bakt.*, Abt. I, Orig., **58**, 120.  
 SCHMIDT, H., 1913, *J. Hyg., Camb.*, **13**, 291, 314; 1914, *ibid.*, **14**, 417, 437.  
 SCHNEIDER, R., 1908, *Arch. Hyg.*, Berl., **65**, 305.  
 SELIGMANN, E., 1907, *Berl. klin. Wschr.*, **44**, 1013; 1908, *Biochem. Z.*, **10**, 430.  
 SKWIRSKY, P., 1910, *Z. ImmunForsch.*, Tl. I, Orig., **5**, 538.  
 SPRINGUT, E., 1927, *Z. ImmunForsch.*, **52**, 25.  
 SURFACE, F. M. & ROUTT, G. C., 1913, *J. Med. Res.*, **28**, 441.  
 TAKENOMATA, N., 1924, *Z. ImmunForsch.*, **41**, 431, 508.  
 TANIGUCHI, T., 1921, *Brit. J. Exp. Path.*, **2**, 41.  
 THIELE, F. H. & EMBLETON, D., 1914, *J. Path. Bact.*, **19**, 372.  
 TOKUNAGA, H., 1928, *Zbl. Bakt.*, Abt. I, Orig., **107**, 283, 288; 1929<sup>1</sup>, *ibid.*, Abt. I, Orig., **111**, 470, 478; 1929<sup>2</sup>, *ibid.*, Abt. I, Orig., **114**, 203.  
 TOPLEY, W. W. C., 1915, *Proc. Roy. Soc. B*, **88**, 396.  
 VALLEY, G. & McALPINE, J. G., 1928, *J. Immunol.*, **15**, 313, 325.  
 WEIL, E., 1913, *Biochem. Z.*, **48**, 347; 1921, *Z. ImmunForsch.*, Tl. I, Orig., **31**, 50.  
 WEIL, E. & FELIX, A., 1920, *Z. ImmunForsch.*, Tl. I, Orig., **29**, 24.  
 WHITE, P. B., 1926, *Sp. Rep. Ser. Med. Res. Coun.*, Lond., No. **103**.  
 WHITEHEAD, H. R., GORDON, J. & WORMALL, A., 1925, *Biochem. J.*, **19**, 618.  
 WORMALL, A., WHITEHEAD, H. R. & GORDON, J., 1925, *J. Immunol.*, **10**, 587.  
 WRIGHT, H. D. & MACCALLUM, P., 1922, *J. Path. Bact.*, **25**, 316.  
 YOSHINARE, N., 1922, *J. Path. Bact.*, **25**, 153.

CHAPTER XI. OPSONIC ACTION ; TROPINS.

BY R. MUIR (UNIVERSITY OF GLASGOW).

**Introduction.**

THE results of research since Metchnikoff's work on phagocytosis was first published have been to supply full confirmation of his views as to the importance of the phenomenon, and it is now generally recognized that extracellular destruction of bacteria in infections plays a small part in defence as compared with the destruction effected through the medium of phagocytosis. The close association of active phagocytosis with a state of immunity is of fundamental nature, and thus the conditions regulating phagocytic action constitute an all-important problem. As is well known, Metchnikoff's original view was that the development of active phagocytosis as a result of immunization indicated a change in the activity of the leucocytes—a sort of education or adaptation, such as occurs among unicellular organisms. The demonstration of the possibility of producing a passive immunity by means of an antibacterial serum, however, rendered this view untenable, and he replaced it by another, according to which an antiserum contained substances which promoted the activity of the leucocytes and which he called *stimulines*. The great objection to this latter conception was the *specific* action of a particular serum—its power of leading to phagocytosis only of a particular bacterium ; and, theoretically, the properties of the serum seemed to have a definite relation to the organisms rather than to the leucocytes.

Denys and Leclef (1895) were the first to demonstrate in the case of an antistreptococcal serum the mode of its action in leading to phagocytosis both *in vitro* and *in vivo*. They showed that in the presence of the anti-serum, the leucocytes of a normal animal ingested the cocci as rapidly as did those of an immune animal. On the other hand, in the presence of a normal serum there was absence of phagocytosis, there being again no difference between the leucocytes of the normal animal and those of the immune animal. They therefore concluded that the leucocytes of the immune animal had not themselves undergone any change, but drew their power from the properties of the serum in which they acted. Mennes (1897) obtained similar results with an antipneumococcal serum. He described the rapid phagocytosis, as seen under the microscope, even of chains of the organism by leucocytes in the presence of the antiserum ; while in the presence of normal serum the leucocytes, even when in contact with the cocci, rarely ingested them. Marchand (1898) made a comparative study of the action of normal serum on avirulent and virulent streptococci

*in vitro*, and found that the former were rapidly ingested by the leucocytes, whilst there was practically no phagocytosis of the latter. He excluded the influence of the products of the virulent organisms as a possible factor in preventing phagocytosis, and concluded that phagocytosis depends upon some physical change in the organisms produced by the action of the antiserum.

In view of these and other results Metchnikoff admitted that an antiserum might act by sensitizing the bacteria so as to lead to their ingestion by the leucocytes, and he spoke of the active substance of the serum as a *fixateur* (immune-body). He still held, however, the possibility of an antiserum increasing the phagocytic activity of leucocytes. Sawtchenko (1902) showed that the principles regulating phagocytosis by an antibacterial serum held also in the case of a serum produced by injecting alien red corpuscles. He found that such a serum contained a specific antibody which acted on the red cells both *in vitro* and *in vivo* and led to their phagocytosis. He maintained, however, that the antibody might become incorporated in the leucocytes and lead to a similar result, his idea being that it acted as a sort of intermediary between the leucocyte and the bacteria. The all-important fact, nevertheless, was established that in the course of immunization there may be developed specific antibodies which promote phagocytosis by acting on the corresponding bacteria or red corpuscles.

The important work of Denys and his co-workers did not receive the attention it deserved, and little investigation along similar lines followed for a time. A new interest was aroused in the subject, however, by the observations of Leishman and of Wright and Douglas on the action of normal serum in promoting phagocytosis. Leishman (1902) introduced the method of counting the number of organisms ingested by leucocytes *in vitro*, the number being then taken as a means of estimating the action of the serum in promoting phagocytosis. He made the important observation in the case of a vaccine for furunculosis, that the average number of organisms ingested increased during immunization. Wright and Douglas (1903-4) showed that in the presence of normal serum a great variety of bacteria were phagocytosed by leucocytes, whereas this did not occur, or occurred only in slight degree, when the serum was heated at 55° C. To the substances in the serum which prepare the organisms for phagocytosis they gave the name of *opsonins*. They showed further that bacteria which had been exposed to normal serum and were then heated at a temperature sufficient to destroy the opsonin, underwent active phagocytosis when brought into the presence of leucocytes. Apparently, then, an opsonin produces some change in the bacterium which is not abolished by heating. They failed to find evidence that the opsonins acted directly upon leucocytes, but considered that their observations on this point were not conclusive. In view of what had been previously established with regard to the part played by various types of antibody and by complement in immunity, the question naturally arose as to the

nature of opsonins and their relation to other recognized serum constituents. Beyond showing the rise of the opsonic property in immunization, Wright and Douglas did not deal specifically with this question, but it gave rise in the years following their first publication to a great deal of investigation and controversy, as will presently be described.

Wright introduced the term *opsonic index* to indicate the relation between the phagocytic action of a particular serum and that of a normal serum. The average number of organisms ingested per leucocyte under the influence of each serum in like conditions was ascertained, and the index was obtained by using the number given by the normal serum as denominator and that given by the serum under investigation as numerator. Along with his co-workers he found that in various local infections the opsonic index was below normal, that is, there was a deficiency of opsonin ; that the condition of immunity was related to and roughly proportional to the opsonic index ; and that the latter could be raised by immunization by means of vaccines. On this basis he introduced the method of vaccine treatment of local infections, the treatment being controlled by observations on the opsonic index. This subject, however, is dealt with elsewhere.

With regard to the question as to the relation of Wright's opsonins to other recognized serum constituents, it soon became evident that increase of the phagocytosis-promoting action of the serum is not usually due to increase in the amount of a thermo-labile substance normally present. Neufeld and Rimpau (1904, 1905) showed that in the development of immunity against streptococci and pneumococci the essential feature was the appearance and gradual increase of *thermo-stable* antibody in the blood of the immunized animal, and in collaboration with Hüne, Haendel and others, Neufeld carried out a valuable series of observations on phagocytosis and the conditions affecting it. He showed, further, that this antibody combined with the homologous bacterium and led to its phagocytosis, that its relationship was a specific one like that of other antibodies, and he held that it was distinct in nature from antibodies already recognized. To it he gave the name of *bacteriotropin*. There thus arose controversy as to the relation of the two types of substance described—viz. opsonin and bacteriotropin—their thermo-lability and their specificity.

Leishman, along with Harrison, Smallman and Tulloch (1905), in carrying out Wright's procedure in the case of immunization against typhoid, found it necessary to heat the serum in order to avoid the occurrence of bacteriolysis, and they showed that when this was done the state of immunity was associated with the presence of a thermo-stable substance which induced phagocytosis. Leishman (1905) obtained a similar result in the case of immunity against *Br. melitensis*, both in the human subject and in animals, and in view of their thermo-stability considered that the antibodies in the two cases were different from Wright's

opsonin. He spoke of them as 'stimulins', supposing that they might correspond with the *stimulines* or *fixateurs* described by Metchnikoff. This view he afterwards abandoned and there seems to be no doubt that the antibodies in question are of the same nature as Neufeld's bacteriotropins.

Dean (1905) made a careful comparative study of a normal and an immune serum, and showed that they differed chiefly in this respect, namely, that on heating at 55° C. the normal serum lost almost all its opsonic property, whilst in the case of the immune serum the opsonic action, though somewhat reduced, still survived to a considerable extent. Accordingly the two types of sera differed in the amount of thermo-stable opsonin present. He saw in this a close analogy to the action of immune-body *plus* complement, and he afterwards (1907<sup>1</sup>) worked out this view more fully and showed that the slight opsonic action of a diluted immune serum could be enhanced by the addition of normal serum; that is, that complementing occurred.

From this historical survey and other observations, two serum constituents, one thermo-labile and one thermo-stable, would appear to be concerned in rendering bacteria sensitive to phagocytosis, and we may next consider the properties and relations of the two substances.

### Opsonic Action of Normal Serum.

The discussion of the subject may be premised by stating that the opsonic action of normal serum is of a relatively general nature. Wright and Douglas (1903) showed that it is exerted against a great many bacteria on which the serum has no bactericidal action and found that of a large number of bacteria tested, only *C. diphtheriae* and *C. xerosis* were not susceptible. Different strains of bacteria, however, vary in this respect and it may be noted that *C. diphtheriae* has been found by other observers to be susceptible to phagocytosis. This was found to be the case by Tunnicliff (1908), Lindemann (1910-11) and Ohkubo (1910). One cannot say that any species is in all states insusceptible, and it is important to recognize that virulence is an important factor. Marchand (1898) showed that an avirulent strain of streptococcus susceptible to phagocytosis under the influence of normal serum became insusceptible when its virulence was raised by *passage*, and similar results have been established in the case of pneumococcus and the anthrax bacillus (p. 373). Ohkubo noted also that avirulent strains of *C. diphtheriae* are more actively phagocytosed than virulent strains. Apart from such exceptions the opsonic action of a normal serum is exerted in a general fashion, and this constitutes an important point for consideration. Theoretically, however, this fact does not exclude the possibility that the serum may contain a number of constituents more or less specific for particular organisms. There are now two subjects to be considered, viz. (1) the question of specificity of opsonins and (2) their nature and mode of union with the bacteria.

## THE QUESTION OF THE SPECIFICITY OF NORMAL OPSONIC ACTION.

Wright and his co-workers showed how in the process of immunization there might occur a specific rise in the opsonic content. They recognized also the existence of a thermo-stable opsonin in heated tuberculous serum, and found that it could be entirely removed by treatment with tubercle bacilli. Wright and Reid (1906) found further that this opsonin present in heated serum is destroyed by heat when the serum is sufficiently diluted, and concluded that it does not differ with respect to its resistance to heat and sunlight from the opsonin which is found in normal serum. One would rather infer from their papers, though this is not expressly stated, that they believed the rise in opsonic power to be due to the increase of one particular substance. They did not consider in detail the possible differences in specificity between a thermo-labile and a thermo-stable element. The subject of specificity was investigated by Bulloch and Western (1906), who found that treatment of normal human serum with tubercle bacilli leaves the opsonic power of that serum for *Staphylococcus aureus* almost intact, whilst opsonic power for tubercle bacilli is completely removed, and conversely, when the serum is treated with staphylococcus the opsonic action on tubercle bacilli is little affected. Further, when the opsonic power for staphylococcus is removed by treatment of the serum with this organism, the opsonic power for *B. pyocyanus* is preserved. They concluded that the serum largely retains its opsonin for the microbe with which it has not been digested.

Absorption tests carried out by other observers, however, gave opposite results as regards general specificity. Simon, Lamar and Bispham (1906) compared the absorptive powers of *Staphylococcus citreus*, *V. metchnikovi*, *B. coli*, &c., and found that treatment of normal serum by one organism removed the opsonic effect, not only for that organism, but also for the others ; that is, that there was an absence of specific combination. Instead of using Wright's method for the estimation of the opsonic action, they diluted the serum till the opsonic effect was slight and then counted the percentage of leucocytes which contained the organisms. They showed also that opsonin could be removed from the normal serum in varying degree by carmine, platinum black, precipitated chalk, &c. Using *Staphylococcus aureus* as a test, Muir and Martin (1906) found that the opsonic property of rabbit's serum was removed by emulsions of various organisms. At the same time the staphylococcus removed the opsonin more quickly and thoroughly than the others. Treatment of the serum with the staphylococcus also removed the opsonin for the tubercle bacillus. Levaditi and Inman (1907), with similar methods, failed to find evidence of specificity and they showed also that treatment of a normal serum with cellular debris annuls its opsonic action. Neufeld and Hüne (1907) obtained a similar result by the use of yeast ; and other examples might be quoted. Negative results as regards specificity are recorded by Russell (1907), Klien (1907) and others.

On the other hand, Macdonald (1906) found that on treating fresh human serum with pneumococci, the separated serum had lost all its opsonic action on pneumococci, whilst there was little diminution of its action on staphylococci ; the same serum after further treatment with staphylococci had lost almost all its opsonic action on staphylococci, but only a small part of that on tubercle bacilli. Rosenow (1907) records experiments which give support to the view as to normal opsonins having specific character. Hektoen (1908) also found that human serum heated at 60° C. on being treated with staphylococci loses the specific action for them but not for pneumococci, and conversely. This was shown by suitable reactivation with fresh serum. He came to a similar conclusion with regard to haemopsonic effect. In recent times, Bull and McKee (1921) have found that in chicken's serum there are particular protective substances for each type of pneumococcus and that these probably act as opsonins. So, also, Sia (1928) absorbed cat's and pigeon's sera with three types of pneumococci and found that absorption with the one left most of the opsonic effect for the others—there was not a general opsonin for all three types.

When we review the results obtained with regard to specificity there is seen to be considerable discordance. This may be due to the amount of bacterial suspension employed for absorption, but it is probably in the main due to variations in the characters of different sera. On going through the various papers we note that distinct evidence of a specific element has been obtained especially when human serum has been used for the test, and this may be of importance. The discrepancy in results, however, is more apparent than real and two facts may be said to be established : (a) The first is that substances which confer a certain amount of specific opsonic action may be demonstrated in small amount in normal sera, and more readily in some sera than others. These appear to vary in their thermo-lability in different cases. (b) The second fact is that there is present in normal serum a thermo-labile substance in considerable amount which exerts opsonic effect, but is without specific properties. We have now to consider the nature of this thermo-labile substance.

#### COMPLEMENT AND OPSONIC ACTION.

The general absorption of normal opsonin by various bacteria, organic and inorganic particles, &c., corresponds with the action of such substances on complement, first shown by v. Dungern (1900), and thus the possibility that normal opsonin is complement or a body closely allied to it was suggested. Dean (1905), in his first paper, stated this as likely and later adopted it definitely, as will be referred to below. In view of the well-established fact that complement is fixed by a great many combinations of antigen *plus* antibody, Muir and Martin (1906) investigated the effect of such combinations on the opsonic properties of normal serum. For this purpose they used what they called 'powerful complement absorbers',

namely, sensitized bacteria, sensitized red corpuscles (heated so as to obviate lysis) and a serum precipitate (serum *plus* antibody); and in every case they found that the opsonic property was practically abolished just as it was by heating at 55° C. for an hour. In contrast to this, they found that similar treatment of a heated immune serum did not in any way diminish its opsonic property (*vide infra*). Levaditi and Inman (1907), in a series of papers, made a comparison between the opsonic and complementing properties of normal sera, and found that they corresponded. To mention one example, they found that aqueous humour was practically devoid both of complement and opsonin, but that the fluid freshly formed in the eyeball to replace that which had been drawn off contained a small amount of both complement and opsonin. Levaditi and Kœssler (1907) showed that anticomplement acts also as anti-opsonin, and Dean (1907<sup>1</sup>) independently obtained a like result. The anticomplementary action is now known to be really due to fixation of complement by serum-antigen *plus* antibody, but this does not affect the significance of these observations as it applies to both properties.

Browning (1908), making use of an observation of Sachs and Teruuchi (1907) which showed that the haemolytic complement of guinea-pig's serum is destroyed to a large extent by diluting with from 5 to 10 volumes of water and then incubating at 37° C., obtained a confirmatory result with regard to the normal opsonin. He found that while the opsonic action of normal serum is destroyed equally with the haemolytic action by this method, the opsonic action of heated immune serum suffers no deterioration. Noguchi (1907), without entering into the general question as to the identity of normal opsonin and complement, found that they corresponded in certain physical properties. Both can be dried at 23° C. without having their properties destroyed. In the dried condition they can withstand high temperatures, even 135° C., and they regain their thermo-lability when the serum is redissolved.

Observations have been made on the relations to opsonic action of the fractions of complement obtained by precipitation (Chapter X). Hata (1908) found that when complement was split there was a close correspondence in the behaviour of the fractions in opsonic action and in haemolysis. Neither end-piece nor mid-piece was active by itself, but they exerted opsonic effect when used together. The results of Ledingham and Dean (1912), though not so clear-cut, are of similar nature. They ascertained that end-piece may have slight opsonic action by itself, probably due to traces of mid-piece, whilst mid-piece was inactive. Combination of the two fractions produced opsonic effect, but rarely up to the original value of the serum. Ledingham (1912) obtained an end-piece from normal guinea-pig's serum which was opsonic against the typhoid bacillus, and used it in an investigation on the mechanism of phagocytosis. He found that both the combination of opsonin with suspension of the bacilli and also the actual phagocytosis of the opsonized organisms, proceeded in a manner corresponding to a process of adsorption.

Zinsser and Carey (1914) found that, while neither end-piece nor mid-piece was by itself effective, on treating the end-piece with weak alkali the opsonic action was in part restored, whereas there was no similar reactivation of haemolytic function. They suggested that the difference may be due to the fact that complement combines directly with bacteria but not with red corpuscles.

It has been recently shown by Gordon, Whitehead and Wormall (1926) that complement contains a fourth component which is destroyed in almost a specific manner by ammonia, the other components being unaffected, and which is also relatively thermo-stable, resisting a temperature of 66° C. On further investigation (1929) they found that treatment of fresh serum with ammonia removes the haemolytic action but does not appreciably affect the opsonic power ; and, further, that absorption of the serum with bacterial suspension sufficient to remove the opsonic and haemolytic properties does not remove the fourth component. They accordingly conclude that the fourth component, whilst necessary for haemolytic and also for bactericidal effect, is not essential for the opsonic action of normal serum.

Another phenomenon bearing on the part played by complement in opsonic action is that described by Friedberger and Hartoch (1910). They showed that if bacteria are treated with a heated serum and thereafter with the corresponding antiserum (precipitin), the opsonic action of a normal serum is much increased. This is apparently due to the fact that the precipitate formed on the surface of the bacteria leads to the fixation of an increased amount of complement and thus to increased opsonic effect. The phenomenon corresponds with the enhancing effect of corresponding procedure in the case of haemolysis, previously described by Friedberger and Moreschi (1908).

#### THE COMPLEMENTING OF NATURAL IMMUNE-BODIES.

Admitting then that the presence of specific substances concerned in opsonic effect can be demonstrated in normal serum, we have next to inquire whether, according to the scheme of bacteriolysis and haemolysis (immune-body *plus* complement), they lead to the union of non-specific complement-opsonin. It has been established that that is the case. The principles employed have been those worked out in the case of haemolysis —the relative resistance to heat of immune-body as compared with complement, and the union of immune-body at a lower temperature (p. 303). For example, Dean (1907<sup>1</sup>), using rabbit's serum and *B. typhosus*, found that the phagocytic index by the conjoint action was greater than the sum of indices of the two separately. He obtained a similar result on complementing diluted immune-serum. Cowie and Chapin (1907-8) found that normal human serum contains an opsonin for *Staphylococcus aureus* which has a similar dual constitution. A heated normal serum may have its opsonin power restored by the addition of diluted unheated serum ; and staphylococci treated with heated serum and then washed

are much more easily opsonized by normal serum than the same organisms untreated. Neufeld and Hüne (1907) came to a similar conclusion from the study of the phagocytosis of various bacilli, and Neufeld and Bickel (1908) obtained a corresponding result in the case of anti-erythrocyte sera. Eggers (1908), using avirulent pneumococci, demonstrated a similar complementing action in the case of normal serum. Among others who have obtained positive reactivation results by means of fresh serum may be mentioned Hektoen (1908), Rosenthal (1909<sup>2</sup>), Böhme (1908), Bürgers and Meisner (1911). It must be recognized that complementing is often difficult to demonstrate and negative results are not infrequent. Fornet and Porter (1908-9), for example, failed to find evidence that true reactivation of heated normal serum occurred. According to Rosenthal (1909<sup>2</sup>) the complementing effect is best obtained if the organisms are first treated with the heated serum and then for only a short time with the fresh serum in diluted form.

In connection with this matter of complementing, it is well that the difficulty of the subject should be kept in view. Some writers appear to suppose that the properties of a normal serum correspond with those established by Bordet and by Ehrlich in the case of a haemolytic serum—combination of immune-body at 0° C., non-union of complement at 0° C., thermo-lability of complement at 55° C., and thermo-stability of immune-body at this temperature. These properties are seen distinctly and with regularity only in the case of artificially prepared haemolytic sera, whilst in the haemolytic action of a normal serum the condition may be quite different. A natural immune-body may be relatively thermo-labile and little of it may combine at 0° C.; for example, the normal lysin of ox-serum towards guinea-pig's corpuscles (p. 313). It is thus sometimes extremely difficult to demonstrate the dual constitution of a normal lysin. In the case of bacteria there is the additional complication that sometimes complement may be taken up by them at 0° C. For example, according to Neufeld and Haendel (1908<sup>1</sup>) cholera vibrios treated with complement-binding antibody fix haemolytic complement at 0 to 3° C. but no bactericidal complement.

The difficulties in analysing the opsonic effect of a normal serum are thus apparent, and it is not surprising that the results of absorption of opsonic substances at different temperatures have varied. We may give some examples of this. Bulloch and Atkin (1904-5) found that much of the opsonic effect of normal serum for cocci was removed by treating the serum with the organisms at 0° C., so also Löhlein (1906<sup>1</sup>); Browning (1908) obtained a similar result and showed, in addition, that a certain amount of haemolytic complement was taken up, though a much greater amount of the latter was absorbed at higher temperatures. Accordingly in this case, if a normal specific opsonic substance were present and fixed at 0° C., we have also the fact that non-specific complement was absorbed at the same time at this temperature. On the other hand, Fornet and Porter (1908-9) found that practically no normal opsonin was fixed by

bacteria at 0° C. provided that sufficient care was taken not to allow the temperature to rise during centrifuging. Dean (1905) found relatively little absorption of normal opsonin (human) for staphylococci at lower temperatures, and Ledingham (1908) obtained a similar result in the case of tubercle bacilli and staphylococci. It is to be noted, however, that although he detected little diminution in the opsonic property of the serum by treatment at 0° C., the organisms used for treating the serum were opsonized. He found that the increase of phagocytosis in a mixture of fresh serum, leucocytes and cocci, which occurs as the temperature rises, is essentially due to the slower combination of the opsonin with the bacteria at lower temperatures, and that once the organisms are opsonized to a suitable degree, ingestion of them by the leucocytes takes place with equal rapidity at temperatures from 18 to 37° C.

#### NORMAL TROPINS.

The establishment of the fact that the opsonic action of a normal serum may occur in a manner corresponding to bacteriolysis does not, however, exhaust the possibilities. As will be described below, the opsonin (tropin) of an immune serum is not only thermo-stable and specific but acts by itself (that is, in addition to any action of immune-body along with complement). The question thus arises whether there is not a homologue in normal serum which likewise acts by itself—a normal tropin. This is theoretically likely to be the case, as it is a general law that the substances and the properties of an immune serum are the representatives and further developments of those in a normal serum. It has been shown that when a normal serum has been heated at 55° C., there may still remain a certain amount of opsonic action. This is a fairly common occurrence, and the heated sera of different animals vary much in respect of the amount of residual opsonic action towards different bacteria. Dean (1905), for example, showed that horse's serum, after being heated at 60° C., had a considerable opsonic effect both on *B. typhosus* and *B. dysenteriae*. He further made the very important observation that treatment of bacteria by a heated normal serum prevented them from taking up the opsonic substance from a heated immune serum. This, of course, indicates that the normal serum contains a tropin which is the homologue of the immune tropin and has the same combining affinity. Other examples of the occurrence of normal tropins are given by v. Gruber and Futaki (1906), Neufeld and Hüne (1907), Clark and Simonds (1908), Fornet and Porter (1908-9) and others ; examples will be found also in Wright and Douglas's (1904) account of their experiments. We may thus conclude that the representatives of normal tropins are not infrequently present, and it is quite likely that some of them may be more sensitive to heating than the corresponding immune-tropins, as has been found to obtain in the case of natural haemolytic immune-bodies (p. 313). According to Rosenthal (1909<sup>a</sup>) the opsonic effect produced by normal tropins occurs more slowly than that in which the action of

complement is concerned. It may be added that it has been shown that a normal serum may contain tropins for red corpuscles—haemotropins (Wakelin Barratt, 1905 ; Neufeld and Töpfer, 1905). It will be seen from what has been stated that any *specific* action displayed by a normal serum depends upon the presence either (*a*) of an immune-body which requires complement in order to be effective, or (*b*) of a substance which opsonizes by itself—that is, a normal tropin.

#### NON-SPECIFIC OPSONIC ACTION OF COMPLEMENT.

A further question is whether complement unites with bacteria directly and opsonizes them. This question is rarely discussed in quite definite terms, but an answer in the affirmative is involved in the conclusion arrived at by many as to non-specific opsonic action. It is known that organic and even inorganic particles of various kinds absorb complement from normal serum and become opsonized in consequence, and there is evidence that bacteria do the same. Although a small amount of natural immune-body may be present in the serum, this seems quite insufficient to explain the large amount of complement which bacteria may take up. A certain amount of suspension of a bacterium (e.g. *B. coli*) removes from a normal serum a definite amount of opsonic effect as tested on another organism (e.g. *S. aureus*). This amount may be increased either by (*a*) increasing the amount of *B. coli* suspension or (*b*) adding a small quantity of *B. coli* antiserum, which leads to increased union of complement. By either absorption method the opsonic action of the treated serum may be reduced almost to nil, and the result indicates that absorption through the medium of immune-body can be replaced by direct absorption by bacteria (see also Gordon, Whitehead and Wormall, 1929). To deny the direct union and opsonic action of complement would involve the supposition that in each case there was a sufficiency of immune-body to fix practically all the complement of the serum. The experimental results supply no evidence of this ; natural immune-body concerned in opsonic action is detectable only in small amounts and the same is true of bactericidal and haemolytic immune-bodies of normal serum. In the absorption of a serum by bacteria alone the opsonic and haemolytic properties diminish together and ultimately may fall practically to nil. But the case is quite different when the serum is treated with red corpuscles, the fall of complement depending solely on the amount of natural immune-body present in the serum, and this amount leads only to the union of a fraction of the complement present in the serum. v. Gruber (1909), in his review of the whole subject, adopted a view similar to that here expressed.

#### SPONTANEOUS PHAGOCYTOSIS.

As Wright and Douglas showed, most organisms require the presence of fresh serum in order to become susceptible to phagocytic action. This is a general law, but exceptions are met with. Sometimes phagocytosis occurs in normal saline in the absence of serum constituents, and is then spoken of as *spontaneous phagocytosis*. This is met with especially

amongst avirulent strains, for example avirulent typhoid and cholera strains (Neufeld and Hüne, 1907), avirulent pneumococci (Neufeld, 1913), *B. pyocyaneus* (Russell, 1907). It is seen also in the phagocytosis of various inert particles (below). There is, however, no general law, as, while some avirulent strains of bacteria undergo spontaneous phagocytosis, most require the action of normal serum. Spontaneous phagocytosis, moreover, is sometimes exhibited in the case of virulent pathogenic organisms. Wright and Reid (1906) showed this in the case of *B. tuberculosis*, and further that spontaneous phagocytosis could be distinguished from that due to the action of serum, by the fact that the former did not occur in higher salt concentration, e.g. 1·2 per cent. A remarkable example of spontaneous phagocytosis is sometimes met with in the case of virulent anthrax bacilli, provided that they are in the non-capsulated state (p. 373). Löhlein (1905) showed that in addition to anthrax bacilli, cholera vibrios of moderate virulence and also various strains of streptococci and *B. coli* may undergo spontaneous phagocytosis.

Of spontaneous phagocytosis no definite explanation has been afforded. It is met with from time to time and may be regarded as due to an accidental occurrence of that state of the bacterial envelope which leads to phagocytosis. There is no satisfactory evidence that it is due to liberation of complement from the leucocytes as has been supposed by some. Were this the case, as Neufeld points out, it should act on avirulent bacteria generally. Nor is there any satisfactory evidence that leucocytes set free any other substance which exerts opsonic action. The physical conditions affecting spontaneous phagocytosis were investigated by Schütze (1914). He showed that the amount of phagocytosis was distinctly increased by the addition of weak acid, whereas weak alkali had no such effect. In neutral suspensions both cocci and leucocytes were found to possess a negative electric charge, and he suggested that on the addition of acid both cocci and leucocytes, owing to their possessing adsorbed protein coats, lose a certain amount of their negative charge, and thus the forces keeping them apart are diminished.

#### OPSONIC ACTION ON INERT PARTICLES.

It has long been recognized that complement is absorbed directly by various particles, organic and inorganic. Simon, Lamar and Bispham (1906) showed that the same obtained with regard to normal opsonin and, further, that the particles used for absorption became opsonized as a result, this being most marked in the case of powdered albumin, charcoal and flour. Milk globules also were found to be subject to opsonic effect. Rosenthal (1909<sup>1</sup>) obtained similar results with various substances. He used especially charcoal and showed that the opsonic action on it is destroyed at 55° C. just like that on staphylococci. Porges (1909) studied the phagocytosis of various particles, but especially starch granules, using 2 per cent. sodium chloride solution as a medium in order to avoid spontaneous phagocytosis, which tended to occur when normal saline was

used. Like Rosenthal he found that normal serum exerted opsonic action and that this was destroyed by heating at 55° C. Shattock and Dudgeon (1908) showed that melanin granules were similarly opsonized by normal serum. In the case of milk globules and melanin the opsonic power of the serum can be increased by injection, and antibodies are developed in the serum (p. 371). Porges obtained negative results on injecting starch, and we know of no positive results obtained with any of the other substances mentioned.

As to the *modus operandi* of normal serum in opsonizing organic and inorganic particles, information is lacking ; all that we can say is that there is apparently a deposit of protein containing complement on their surface. With regard to spontaneous phagocytosis of inert particles, the fact that it is inhibited by increased concentration of the salt-content which does not affect ordinary phagocytosis, indicates that the former may be of different nature from the latter.

#### DISTRIBUTION OF OPSONIC SUBSTANCES.

Opsonic action in normal conditions may be said to be essentially a property of the blood. Simon, Lamar, and Bispham (1906) made extracts of various organs in normal saline and found them to be without opsonic effect. The only exception was the bone-marrow, the extract of which had a slight opsonic action which they attributed to the contained blood. They found that the content in opsonin of the chyle was 60 per cent. of that of the blood, while that of transudates, e.g. hydrocele fluid, was much lower. No opsonic property was detectable in the cerebrospinal fluid, seminal fluid or milk. Negative results as regards the cerebrospinal fluid were obtained by Böhme (1908) and, as regards the aqueous humour, by Levaditi and Inman (1907). v. Gruber and Futaki (1907) found that the opsonic action of the fluid of subcutaneous tissue was weaker than that of the blood. According to Opie (1907) the serum of a purulent exudate, produced by a sterile irritant, may contain no opsonin for staphylococci. This is probably due to the opsonic complement being absorbed by the degenerated tissue elements. The foetal blood contains less opsonic property than the maternal (Bürgers, 1910). Tunnicliff (1910) showed that normal opsonins for various organisms were less at birth than in the adult stage. There is a fall in the opsonic content for some months after birth and then a gradual rise to the normal level occurs, this being reached at the end of the second year. Tobler (1924) records somewhat similar results.

#### INHIBITION OF PHAGOCYTOSIS.

In certain instances an inhibitory action exerted by a heated normal serum on normal opsonic effect has been described, phagocytosis in such cases being more marked in normal saline than in the heated serum. Rosenthal (1909<sup>a</sup>) observed this in the case of various particles—carmine, carbon, and soot—as well as in the case of staphylococci ; and Porges (1909), using starch grains, noted a similar phenomenon when 2 per cent.

saline was used as the medium. Fornet and Porter (1908-9) found that there are two kinds of opsonin in normal serum and also an antagonistic substance which acts on one of them. The nature of such inhibition is obscure, but apparently the heated normal serum produces some physical change on the surface of the particles or bacteria, since after being treated with the heated serum and washed they are less readily phagocytized than when untreated (Rosenthal, 1909<sup>2</sup>; Porges, 1909). As already mentioned, phagocytosis *in vitro* is occasionally inhibited by products diffused by bacteria when they are in a very virulent state (Ruediger, 1905; Tchistovitch and Yourevitch, 1908); and further, a heterologous serum may have inhibitory action on the leucocytes when the serum and leucocytes are from species widely different zoologically.

Another example of inhibition may be given. Ledingham (1907) showed that heated normal serum through which tubercle bacilli have been passed exerts marked inhibitory action on the opsonic action of fresh serum on tubercle bacilli and staphylococci. This is probably due, as he supposed, to the presence in the heated serum of normal immune-bodies which combine with free receptors thrown off by the tubercle bacilli, and so fix complement, with resulting inhibition of phagocytosis. It is thus seen that the inhibition of the opsonic action of normal serum may be brought about in a variety of ways.

### Immune Sera.

The difference between the opsonic action of a normal and an immune serum can be expressed in quite definite terms. The immune serum is characterized by its containing a varying amount of antibody developed during the process of immunization ; the antibody is specific towards the organism used in the process and is thermo-stable in character. As has been described above, a normal serum may contain a distinct trace of such a specific substance or, on the other hand, none may be detectable. We cannot, however, say that there is always some such normal representative ; and there is evidence that, just as in the case of haemolysins, the normal representative may be more labile than the immune. These antibodies present two types of action : (a) the opsonic effect may be produced by the antibody alone, and (b) the opsonic effect may be due to the interaction of complement along with the antibody. The terms (a) tropin, and (b) opsonic immune-body or immune-opsonin have been applied according to the type of action displayed. For convenience we may speak of two types of antibody, without, however, implying that the two modes of action may not be possessed by the same substance. This subject will be discussed later (p. 369).

### BACTERIOTROPINS.

The specific substances which produce opsonic effect by themselves are in some respects the more important and are now generally known by the name of tropins adopted by Neufeld—bacteriotropins, haemotropins, &c.,

according as they have been developed against bacteria, erythrocytes or other substances. If a fresh antibacterial serum be heated, it may, of course, lose a certain amount of its opsonic action due to destruction of complement. Dean (1905), for example, found in the case of an anti-staphylococccic serum that this occurred in two minutes at 60° C., and that on subsequent heating the opsonic potency remained practically constant for a long period. We may say that the thermo-stability of bacteriotropins corresponds generally with other antibodies—agglutinins, immune-bodies, &c. Differences may no doubt be made out between the different types and these vary in the case of different sera. But, to speak generally, heating at 55° C. for half an hour destroys the complement and leaves the antibody practically unaltered. Like other antibacterial substances, bacteriotropins suffer varying degrees of deterioration at higher temperatures, such as 70° C.

With regard to the specificity of bacteriotropins all observers are in agreement. In illustration the experiments of the writer with Martin (1907) may be quoted. In the case of a heated antistaphylococccic serum it was found that treatment of it with emulsions of different organisms had no effect whatever on its opsonic power for *Staphylococcus aureus*, the only exception being a very slight absorptive effect in the case of *Staphylococcus albus*. In the fresh state the serum may lose some of its opsonic property by such treatment owing to the absorption of complement, but after it has been heated at 55° C. there is no such loss on treatment with heterologous organisms. On the other hand, as we have already described, treatment of a normal serum with such emulsions removes the opsonic effect almost entirely. Russell (1907) obtained similar results with an antityphoid serum, and practically all observers have come to the same conclusion. In fact, experiments of such a kind in the case of immune sera give such a clear-cut result that there is no doubt as to specific properties. When agents which act as complement-absorbers and so markedly diminish the opsonic action of fresh normal serum are applied to a heated antiserum containing a specific bacteriotropin there is no effect on its action. Muir and Martin (1907) showed this by using the various combinations of antigen *plus* antibody mentioned above (sensitized erythrocytes, sensitized heterologous bacteria and serum precipitate)—combinations which removed the opsonic effect almost entirely from a fresh normal serum. The general effect of such treatment is the same as heating at 55° C. ; in the former case the opsonic complement is removed by fixation, in the latter it is, of course, destroyed. Levaditi and Inman (1907) similarly found that an 'anticomplement' had no effect on immune-opsonin. They considered that there is a close relationship between tropins and immune-bodies but did not assert their identity. We may add that we know of no example where increased specific opsonic action developed by immunization has been shown to be due to increase of a thermo-labile substance of simple constitution present in a normal serum, as was supposed by some of the earlier workers.

When we speak of the *specificity* of bacteriotropins, the term is to be used in the same sense as in the case of other antibodies—that is, a bacteriotropin may have some action on bacteria allied to the bacterium against which the antiserum has been developed. This subject has not been worked out with the same fullness as in the case of agglutinins and other antibodies, but sufficient has been established to indicate that the same general law obtains. What may be called group bacteriotropins have been established in the case of the typhoid and hog-cholera groups. An antityphoid serum powerfully opsonic for the typhoid bacillus may have some opsonic effect on paratyphoid bacilli, and conversely. Ungermann (1910), using an antipneumococcic serum which was preventive against infection with various pneumococcus strains and had an opsonic action on them, found an atypical or 'serum-fast' strain on which the serum had no effect. In a similar way the antiserum to the atypical strain had no opsonic effect on the other strains. This is in accordance with what has since been worked out more fully with regard to types of pneumococci.

A striking feature of an immune serum is the high degree to which it may be diluted before it loses much opsonic action. For example, Dean (1907<sup>1</sup>) found that an antistaphylococcic serum, when diluted 32 times with saline, lost only 25 per cent. of its opsonizing power, whereas a normal serum diluted to the same extent lost 90 per cent. The effects of dilution were studied also by Marshall (1907), who showed that an immune serum, in contrast to normal serum, might be diluted over 30 times without suffering any marked fall in its opsonic power. This property is well exemplified in the case of antimeningococcic serum, which may sometimes be capable of being diluted several thousand times without being rendered inactive (Neufeld, 1913). Jobling (1909) used the opsonic method for estimating the potency of such serum and adopted a titre of 1 : 5,000 as a standard of efficiency—that is, the serum ought still to have opsonic effect on being diluted 5,000 times. Our own experience is that when tested on a given organism the action of a normal serum, where opsonic complement is concerned, is often more intense than the action of a heated immune serum due to bacteriotropins. The former may give a higher phagocytic count than the latter when they are used in the undiluted condition, but on dilution the opsonic effect of the normal serum is much more quickly lost.

#### IMMUNE-BODIES IN OPSONIC ACTION.

It is thus seen that a bacteriotropin is a thermo-stable specific antibody which, by itself, produces the essential change in a bacterium, resulting in susceptibility to phagocytosis. The opsonic effect of an immune-serum may, however, in addition be due in part to its content of immune-body which does not by itself opsonize the bacteria, but which acts by leading to union of complement. As has been shown above, complement produces opsonic effect, and as additional complement combines through the

medium of immune-body, this effect will be increased. (I repeat that the term complement is used simply as meaning the labile substance of a normal serum which is fixed by the combination antigen *plus* antibody; whether bacteriolytic, haemolytic or opsonic complements are identical or not is for the present purpose immaterial.) In accordance with this we find that the opsonic effect of an unheated immune-serum is reduced to the same extent by treatment with a complement-absorber as by heating at 55° C., a procedure which, of course, does not affect bacteriotropins present. Furthermore the complementing of an immune-body by normal serum can be demonstrated in opsonic action. In testing this the heated serum must be diluted till its opsonic effect is almost abolished, and then the opsonic effect is tested as regards (a) diluted antiserum alone, (b) fresh serum alone, and (c) diluted antiserum *plus* fresh serum. It may be necessary to dilute the normal serum also. Dean (1907<sup>1</sup>) showed by such a procedure that the phagocytic count by diluted antityphoid serum *plus* complement exceeded the sum of the phagocytic counts of each by itself. Similar complementing of immune-body has been shown by Neufeld, Levaditi and Inman, and others. In order to avoid any possibility of some mode of action by summation of effects, Browning (1908) adopted the following procedure. He treated staphylococci first with their antiserum diluted, and then washed them free of serum; on the addition of fresh serum the increased opsonic action due to complementing occurred; but when the reverse order was adopted—when the staphylococci were treated first with complement and washed free of serum and then the same amount of diluted antiserum added—no complementing effect resulted. This is in conformity with what is known as to the action of immune-body and complement and appears conclusive on the subject.

Neufeld and Bickel (1908) showed also in the case of a haemolytic antiserum that the participation of immune-body in opsonic action could be demonstrated. The method found successful was to use minute doses of the heated serum insufficient for lysis, the addition of complement then leading to phagocytosis of the corresponding erythrocytes. Hektoen (1909) obtained a similar complementing result.

While the part played in any opsonic action by immune-body can often be demonstrated, this, however, is not always the case. Neufeld and Hüne (1907) failed to demonstrate any action of immune-body in producing opsonic effect in the case of antistreptococcal and anti-pneumococcal serum. They found also that in an antiparatyphoid serum only tropins were concerned. Fornet and Porter (1908-9), in a similar manner, failed to demonstrate by reactivation the presence of immune-body in various antisera. Other similar results are recorded.

The relative importance of the two modes of action of an antiserum in promoting phagocytosis has been variously interpreted, and there has also been discussion as to whether two distinct types of antibody are concerned. Some writers, for example Dean and especially those of the French school, Levaditi, Sawtchenko and others, have laid special stress

on the opsonic action of immune-body with complement. Others again, for example Neufeld and his co-workers, while acknowledging the participation of complement through the medium of immune-body, consider that tropins are distinct substances, and that their action is the more important so far as protection of the organism is concerned. They are certainly justified in laying much weight on the fact that the tropins can produce their effects when complement is deficient or absent. Thus complement is relatively less abundant in the tissue juices than in the blood, whilst in the cerebrospinal fluid and anterior chamber of the eye it is practically absent. The relatively high concentration of tropins which may be present in an immune serum is also a fact of importance.

All are agreed that there is no rule as to the relative proportion in which the two properties are present in antisera. Sometimes immune-bodies, as tested by complement-fixing action, and tropins are present together in large amount, but the greatest variation in their proportion is met with. An antiserum may contain immune-body and when heated may have no opsonic action ; or again, tropin may be present and it may not be possible to demonstrate increased action by the addition of complement (Neufeld and Hüne, 1907 ; Neufeld, 1910). The characters of any antiserum depend upon the individual animal and on the particular antigen used ; and the variations in each may modify the result considerably. Even in what we might regard as similar conditions the properties of the serum vary. For example, in the case of antiserum obtained from the rabbit to guinea-pig's corpuscles, Neufeld and Töpfer (1905) found no tropins but much immune-body, yet Sawtchenko (1902) found a similar serum to possess marked tropic effect and employed it in his investigations on phagocytosis. The most satisfactory method to study the question would appear to be to estimate the content of different antibodies in the serum of one animal in the course of immunization. This has been done by various observers, and the general result is that although there may be a fairly close correspondence between the curves of immune-bodies and tropins, considerable variations are met with. This procedure was carried out in the case of haemolytic sera by Neufeld and Bickel (1908), who found that the serum drawn at different times gave phagocytosis and lysis in varying proportion. They considered that the results could not be reconciled with the view that the two effects are due to one antibody. Hektoen (1909<sup>2</sup>), using the same method, came to a like conclusion, though his curves of immune-body and tropins have a certain similarity. Henderson Smith and Brooks (1912) made a detailed study of the subject and found important differences as regards mode of production between bactericidal antibodies and those concerned in phagocytosis.

In view of these and other results on this subject, it is held by many writers that tropins and immune-bodies are distinct substances. Such a conclusion would be justified provided only that each antibody was of definite type and retained its characters unchanged during the process of immunization. It has been shown, however, that an antibody, for

example a haemolytic immune-body (p. 306), may undergo qualitative alterations at different stages of immunization and accordingly it is possible, indeed probable, that an opsonic antibody behaves in a similar way, and that it may vary at different times as regards the relative proportion in which it bears the two modes of opsonic action, direct and indirect. According to such a view the variations in the opsonic properties of different antisera might be explained. Against this conception the objection is sometimes made that it supposes a new type of antibody ; but this seems to have little weight in view of the small number of types of antibody recognized and our imperfect knowledge regarding them. Further, in the case of antibodies to proteins (precipitins) the combination with the homologous antigen does both produce a physical change resulting in precipitation and also leads to the union of complement. This corresponds in a way to a tropin possessing also the function of an immune-body. In connection with this and similar questions there has been too great a tendency to assume that established facts exhaust possibilities, and also to speak as if there were a definite number of serum constituents, each with a well-defined and uniform character.

In summary of this part of the subject, we may say that the all-important fact in relation to immunity is the development of thermo-stable specific antibodies which promote phagocytosis. Their action is partly a direct one on the organisms and partly through the medium of combined complement. In this way we can say that the antibodies concerned are of two types—tropins and opsonic immune-bodies, these varying in proportion in different cases. It is, however, probable that the two modes of action may be possessed by the same substance.

It may be stated as a general law that in the case of *alien cells* and *particulate substances* which act as antigens, the antisera developed by their injection may have opsonic properties. Anti-erythrocyte sera have been specially studied in this respect and their opsonic action has been found to correspond in all essentials with that of antibacterial sera. Various facts have already been incidentally referred to, and for further details reference may be made to the papers of Barratt, Keith, Dudgeon, Hektoen, Neufeld and Bickel. It was shown by Neufeld and Haendel (1908<sup>2</sup>) that milk globules underwent phagocytosis under the action of an antimilk serum, and they found that in this a thermo-stable tropin was concerned. An antiprotein serum likewise exerted opsonic action on globules of olive oil which had been emulsified in the homologous protein and thus coated by it. In such conditions phagocytosis is due to an action on the proteins in the envelopes of the globules. Ledingham (1909) produced an antiserum to granules of hippomelanin and found that it had marked opsonic effect. In this case the antibody was shown to have the constitution of an immune-body ; when the serum was heated its action could be restored by the addition of complement. He did not conclude definitely whether the antibody was one to melanin itself or to some associated protein.

**Phagocytosis in vivo and in vitro.**

A very important question running through all this subject is to what extent phagocytosis corresponds in these two conditions. The subject of protection in the normal state has been discussed elsewhere and it has been shown that phagocytosis is much more important than is direct bactericidal action of the serum. A similar statement applies to the state of acquired immunity ; the number of bacterial species which can be killed by the action of antibacterial sera form a comparatively small proportion. Opsonic action leads to transference of the bacteria to a situation in which they can be more satisfactorily dealt with, and there is ample evidence that they are often destroyed within the phagocytes. This can be often seen *in vitro* by microscopic examination, and it has also been shown by cultural methods in certain instances that serum along with leucocytes has bactericidal action while serum alone is without effect (Rosenow, 1906 ; Ruediger, 1905, 1906<sup>1</sup> ; Hektoen, 1906). Hence arises the importance of the significance of in-vitro experiments in relation to what may be observed in the body and thus to the general question of defence. The subject has been studied in two ways—(a) by comparing the action of a normal serum on avirulent and virulent strains of the same organism, and (b) by comparing the effects of an antiserum with those of a normal serum on the same strain. Most observations have been made by the latter method, but significant facts have been established also by means of the former.

With regard to the action of an antiserum as compared with that of a normal serum, the first and fundamental observations recorded were those of Denys and Leclef (1895) already referred to. They showed that with a streptococcus of virulent type phagocytosis occurred in the corresponding antiserum and not in a normal serum, while they were unable to detect any difference between the properties of the leucocytes of the immune and of the normal animal. These results were confirmed and were found to hold also in the case of antipneumococcic serum by Neufeld and Rimpau (1904), who showed further that the antibody (tropin) combined with the bacteria and not with the leucocytes. These two types of antiserum have been extensively used in investigating the question, and a close correspondence between the in-vitro and in-vivo results has been established. Ungermann (1910) found a strain of pneumococcus which was not opsonized by antipneumococcic serum, but on immunizing an animal with this strain he obtained a serum which had marked effect on it but not on other strains of pneumococci. Antigenic properties of the two strains were thus different, and there was a close parallelism between the opsonic effects *in vitro* and *in vivo*, whilst these effects corresponded also with the protective action of each homologous serum. Neufeld and Hüne (1907) found a similar correspondence between the effects in the two conditions in the case of typhoid and paratyphoid antisera, and Kraus and St. Baecher (1909) in the case of antimeningococcic

serum. Flexner and Jobling (1908) showed that antimeningococcic serum, when used in the treatment of cerebrospinal fever, acted by leading to phagocytosis of the organisms as well as by its antitoxic action ; and clinical investigation has established that in a great many other infections the establishment of immunity is accompanied by an increase in the opsonic properties of the serum. In fact, the development of opsonic antibodies may be said to be the most important factor in antibacterial immunity. But while this is the case, examples of antisera are known, for instance that against anthrax (Chapter VIII), whose protective effect cannot be explained by the opsonic antibodies which they contain.

Examples can also be given where the development of virulence in an organism is accompanied by increased insensitiveness to opsonic action. Mennes (1897) showed this in the case of pneumococci and Marchand (1898) in the case of streptococci—avirulent types are phagocytosed by the action of normal serum whilst virulent types are not. It may be noted that in the case of avirulent types of bacilli subject to bacteriolysis (e.g. *B. typhosus*) the phagocytic count with heated serum may be greater than with unheated serum—this resulting in the so-called ‘reversed ratio’ (Dean, 1907<sup>2</sup>). The phenomenon is apparently due to lysis of the organisms by the complement of the fresh serum. Löhlein (1905) studied various types of streptococci and *B. coli*, and found that whilst their properties varied there was a close resemblance between the results *in vitro* and *in vivo* with the different types. Sometimes, however, virulent strains of organisms are susceptible to the action of normal serum. This is so in the case of the anthrax bacillus, and the matter was investigated by Löhlein (1905), Neumann (1907) and by v. Gruber and Futaki (1907), the results obtained by them being in agreement. These observers showed that when virulent anthrax bacilli, susceptible to phagocytosis *in vitro*, are introduced into the animal body, phagocytosis of them may actually be observed for some time. After some hours, however, a new race of bacilli which are encapsulated appears and there is no phagocytosis of these ; the organisms multiply rapidly and death of the animal follows. The encapsulated bacilli when tested *in vitro* are found to be insusceptible to phagocytosis. This apparent exception to the general rule has accordingly on investigation supplied confirmatory evidence as to the correspondence between the phenomena in the two conditions. Löhlein (1906<sup>2</sup>) and Markl (1908) found that a similar condition held with regard to virulent strains of plague bacilli susceptible to phagocytosis—a preliminary phagocytosis in the body was followed by the appearance of a resistant strain. Tubercle bacilli form a well-known example of pathogenic organisms sensitive to normal opsonic action, and phagocytosis of them is met with in the tissues of a susceptible animal—in this case, phagocytosis, however, is not attended by a condition of immunity. We may accordingly say that work on this subject has gone far to establish the important significance of the results of experiments *in vitro* in relation to what occurs in the living body.

**Intracellular and Extracellular Bactericidal Action.**

It has already been shown that organisms may be opsonized by the co-operation of two substances, an immune-body and complement—in other words, according to the principles obtaining in bacteriolysis and haemolysis—and such a mode of action obtains in the case both of normal and of immune sera. Even, however, when complement is concerned in opsonic action there is no evidence that the change produced in the bacteria is of the nature of a minimal or imperfect lysis, as theoretically might be the case. Again, the view maintained by many of the French school, that extracellular and intracellular destruction are essentially the same, has not been established. According to this view the actual lytic process in either case is due to complement or alexin formed by the leucocytes and acting in one case outside the cells, in the other in their interior. *In vitro* experiments cannot be said to support this. There is no evidence of the setting free of complement by the leucocytes in heated serum or in saline—no replacement of the complement destroyed by heating. Further, when bacteria altered by lysis occur both within and outside of the leucocytes, the appearances show that the lytic change is produced in the organisms before they are taken up. Wright and Douglas (1904) showed this in the case of typhoid bacilli and cholera vibrios. When a fresh serum was used the organisms became spherulated and altered in appearance and similar forms occurred within the leucocytes. On the other hand, when phagocytosis occurred under the influence of a heated serum, the organisms, both extracellular and intracellular, were well preserved. There were no signs that the opsonic effect produced by the serum was completed inside the leucocytes by a lytic action similar to that caused by the complement of fresh serum. It should be noted that Löhlein (1907) described the occurrence of granular transformation of cholera vibrios within the leucocytes in certain cases of spontaneous phagocytosis. But this does not affect the significance of the difference of the results described by Wright and Douglas. Again, when sensitized red corpuscles are taken up by leucocytes they may retain their haemoglobin and appear little changed for a considerable time; there is no rapid lysis such as occurs under the action of a haemolytic serum containing complement. The difference between extracellular and intracellular destruction was insisted on by Neufeld (1908<sup>2</sup>), who found that in extracellular lysis of red corpuscles, bacteria and trypanosomes, stromata (Schatten) were always left, whilst the destruction within the cells, though slower, was complete. In view of all the facts, we accordingly conclude that while an immune-body acts both in lysis and phagocytosis, destruction of the organisms following in both cases, there is not satisfactory evidence that the agent in intracellular destruction is complement or that the source of the complement of the serum is the leucocytes (*vide* leukins, p. 284).

Facts with regard to the behaviour of the leucocytes of different species of animals in phagocytosis have already been given in connection with natural immunity. It is sufficient to recall that leucocytes from a great variety of sources appear to be equally active in heterologous normal sera, although, of course, the homologous serum is the ideal medium, and some foreign sera may have an inhibitory or even injurious effect. The statement applies not only to the leucocytes of mammals and birds but also to cold-blooded animals—frogs, fishes, &c., and even to some invertebrates (Ruediger and Davis, 1907). In connection with our present purpose the important questions are whether there are any differences between the leucocytes of the normal and of the immune animal, and whether a serum antibody acts directly on the leucocytes. It should be noted that the latter question involves not merely increased activity but increased *specific* activity of the cells. Since the phenomena of phagocytosis have come to be studied by *in-vitro* along with *in-vivo* methods, the general results have gone to show that the all-important action of specific antibodies is on the homologous bacteria. Denys and Leclef (1895) found that leucocytes of the normal animal were just as active in phagocytosis as those of the immune animal, and the differences in their behaviour were due to the action of the serum on the bacteria. Wright and Douglas (1903-4) failed to find evidence that opsonins acted on the leucocytes but they left the question still an open one.

It may be said that while there is no substantial evidence that the leucocytes are affected in a *specific* way by opsonic substances they are not to be regarded as a quite indifferent factor. Variations in phagocytic power are found in the leucocytes of normal individuals and also in certain diseases (Glynn and Cox, 1909-10; Boughton, 1910; Rosenow, 1910). Rosenow, for example, found that leucocytes of pneumonic patients were more active towards pneumococci than the leucocytes of normal individuals. But, on the other hand, leucocytes from the cases of other acute infective conditions showed a similar increased activity. (This subject is reviewed by Hektoen, 1911; see also v. Philipsborn, 1924.) Madsen and Wulff (1919) have brought forward important results in relation to temperature. They have found that the maximum phagocytic activity of the leucocytes occurs at the temperature of the person or animal from which they are taken. This holds not only in the case of normal individuals but also in disease. The leucocytes from a case of pyrexia are most active at the temperature of the patient when the blood is drawn. Sawtchenko (1902), from a study of phagocytosis of erythrocytes, considered that the substance leading to phagocytosis could be absorbed by the leucocytes, with the result that there was a specific increase of their activity towards the corresponding erythrocytes. In the same research, however, he found that there was also direct union of antibody with the erythrocyte with opsonic effect. It is to be noted, however, that this view was based on *in-vivo* experiments, and there are other possible explanations of his results. He regarded the opsonin as a sort of go-between

and held that it could come from the side of the leucocyte as well as be attached to the erythrocyte. This view involves ultimately the action on the erythrocyte, and it seems impossible to explain the specific action on leucocytes apart from some such theory. Up to the present, however, it cannot be said that such specific effect on the leucocytes has been satisfactorily established.

### Mode of Action of Opsonic Substances.

It is now universally recognized that the substances which promote phagocytosis, whether they be of the labile non-specific type or of the stable specific type, have a direct action on the bacteria. This has been established alike by *in-vitro* and *in-vivo* experiments. It must be recognized at the same time, however, that in certain cases the bacteria may produce substances which have a prejudicial effect on the leucocytes and thus prevent phagocytosis—the leucocidins and other aggressins are examples of this ; and further, antibodies may in certain conditions be developed to these. But it is quite clear that phagocytosis is not ordinarily regulated in this way. This will be seen from the account already given, but we may add some other facts in illustration. Thus virulent organisms not susceptible to phagocytosis remain still insusceptible after being killed and freed from their products ; in this instance there is no question of the formation of harmful substances which act on the phagocytes. Marchand (1898), at a comparatively early period, showed that in a mixture of virulent and avirulent streptococci only the avirulent were taken up by the leucocytes. And Bordet (1897) showed that when the leucocytes were placed in a mixture of virulent streptococci and *B. proteus*, only the latter organisms were taken up by the phagocytes ; this showed that there was no interference with the activity of the cells. A somewhat analogous example in the case of red corpuscles is given by Neufeld (1908<sup>1</sup>), who used antisera for sheep's and fowl's corpuscles. He found that when the two kinds of corpuscles were mixed and one of the two antisera was added, only the homologous corpuscles were taken up by the phagocytes.

Practically all observers agree that the substance promoting phagocytosis produces some physical change in the bacteria, red corpuscles, &c. This was the view essentially established by the early observations of Marchand (1898) and of Wright and Douglas (1903-4) referred to above. The opsonic agent causes some distinct alteration and its actual presence in the fluid is not necessary after this alteration has been effected. The preliminary change necessary for phagocytosis appears to be chiefly one in the bacterial envelope and to result in increased adhesiveness. For example, Barikine (1910-11) showed that leucocytes may adhere to a glass surface and that when this has occurred, erythrocytes treated with their corresponding tropin adhere firmly to the leucocytes, while untreated erythrocytes exhibit no adhesive property. Levaditi and Mutermilch

(1910) found that there was a corresponding adhesiveness between trypanosomes and the leucocytes in the presence of the antiserum to the former.

The view that susceptibility of organisms to phagocytosis is associated with and due to some change in the bacterial envelope, is supported by well-recognized facts with regard to the relation of capsule-formation to virulence. For instance, Bordet (1897), in studying the effects of virulent streptococci on the guinea-pig, which is not so susceptible as the rabbit, found that when a fatal dose was used the streptococci were at first taken up by the leucocytes, but after a time became surrounded by capsules or areolæ, multiplied rapidly and remained in the free condition. A similar occurrence is well recognized also in the case of the bacilli of anthrax and plague referred to above. The association of capsule-formation and increased virulence in these examples suggests that, when organisms are placed in surroundings unfavourable to growth in the animal body, they often react by changes in their envelope accompanied by increased resistance to phagocytosis. The fact that an opsonic substance acts on the envelope does not exclude the possibility of action also on the substance. In this connection it is interesting to note the observations of St. J. Brooks (1912), who found in the case of plague organisms that the antigen which increased opsonic action is the nucleoprotein content in the bodies of the bacilli. Again, as has been described above, in the case of certain bacilli the organisms contain two types of antigen—the H or flagellar, and the O or somatic. Braun and Nodake (1924), in investigating the relation of these to phagocytosis, find that it is especially the somatic antigen or endoplasm that gives rise to the opsonic antibody and binds it.

The occurrence of actual physical change in the bacterial surface has recently been demonstrated by Mudd, Lucké, McCutcheon and Strumia (1929), in the case of acid-fast bacilli. They found that when these organisms became opsonized, this was accompanied by increased adhesiveness, decrease in surface electrical potential, and a change in surface tension, so that they became less easily wetted by oil and more easily by saline, the last-mentioned change being studied at the interface of the two fluids. Such alterations were found to obtain alike when the organisms were opsonized by normal serum or by unheated and heated immune serum. Attempts to opsonize bacteria by treating them with various chemical substances were made by Marchand (1898) and v. Gruber (1909) but without positive result, and so far as is known to the writer, success has not yet been obtained. As stated, virulent organisms are not rendered susceptible to phagocytosis on being killed, and Neufeld (1913) observed that virulent meningococci when degenerated were not taken up by the leucocytes more readily than those of healthy appearance.

After bacteria have been opsonized, the rapidity with which they are ingested when brought into contact with leucocytes is remarkable. This has been shown in a striking manner by Fleming (1927), who used the recent method of Wright (1926) by which mixtures of leucocytes, bacteria

and serum are placed in capillary tubes and then centrifuged. By this method Fleming has found that the phagocytosis of fully opsonized bacteria is practically complete after centrifuging for about one minute. Apart from such mechanical means, actual contact may occur spontaneously, viz. (a) by physical forces, and (b) by actual movement of the leucocytes towards the sensitized organisms under chemotactic stimulus. Fortuitous contact plays a part but is often quite insufficient to explain the remarkable rapidity of phagocytosis at a suitable temperature. Rosenthal (1909<sup>2</sup>), for example, found that at 37° C. the phagocytosis of opsonized staphylococci was almost completed in a few minutes, the early ingestion taking place at the rate of about seven cocci per minute. It would seem that some aggregating force or sometimes actual agglutination draws the organisms together, and Ledingham (1912) found that at a temperature sufficiently low to suppress amoeboid activity the opsonized organisms tend to collect in rings around the leucocytes, little ingestion occurring until the temperature is raised. Some such phenomenon is essential to explain the rapidity and degree of phagocytosis in certain instances. Adhesiveness between the organisms and the leucocyte protoplasm means lowered surface tension at the point of contact, and Bordet (1920), in his work on immunity, considers that such fine particles as bacteria are merged in the protoplasm quite apart from amoeboid activity. Ledingham (1912) put forward a similar view. The part played by adhesiveness is discussed by Kite and Wherry (1915). The fact that active phagocytosis is mainly exerted by the polymorphonuclear leucocytes, the eosinophiles and lymphocytes playing practically no part, must be referred to the properties of the leucocyte protoplasm. Red corpuscles and other relatively large bodies become adherent to the leucocytes in a similar way and then amoeboid activity is seen ; the protoplasm rises round them and ingestion follows. The nature of amoeboid activity is, however, outside our present subject. [For physical considerations reference may be made to the writings of Rhumbler (1910), Fenn (1921-2) and Ponder (1925-6).]

While the leucocytic movement under chemotactic stimulus is not concerned to any extent in ordinary opsonic phenomena with bacteria, it does sometimes play an active part in phagocytosis *in vitro*. Comandon (1919), for instance, has shown by striking cinematographic methods that in the phagocytosis of starch granules by frog's leucocytes *in vitro* the latter move in straight lines towards the granules and then ingest them, true chemotactic attraction thus being concerned. On the other hand, he found that there is no such attraction in the case of carbon particles, the ingestion of which is a physical phenomenon of capillary adhesion.

#### REFERENCES.

- BARIKINE, W., 1910-11, *Z. ImmunForsch.*, Tl. I, Orig., **8**, 72.
- BARRATT, J. O. WAKELIN, 1905, *Proc. Roy. Soc.*, B, **76**, 524.
- BÖHME, A., 1908, *Münch. med. Wschr.*, **55**, 1475.
- BORDET, J., 1897, *Ann. Inst. Pasteur*, **11**, 177; 1920, *Traité de l'immunité*, Paris, p. 238.

BOUGHTON, T. H., 1910, *J. Infect. Dis.*, **7**, 111.  
 BRAUN, H. & NODAKE, R., 1924, *Zbl. Bakter.*, Abt. I, Orig., **92**, 429.  
 BROOKS, R. ST. J., 1912, *J. Hyg., Camb.*, *Plague Suppl.* **2**, 373.  
 BROWNING, C. H., 1908, *J. Med. Res.*, **19**, 201.  
 BULL, C. G. & MCKEE, C. M., 1921, *Amer. J. Hyg.*, **1**, 284.  
 BULLOCH, W. & ATKIN, E. E., 1904-5, *Proc. Roy. Soc.*, **74**, 379.  
 BULLOCH, W. & WESTERN, G. T., 1906, *Proc. Roy. Soc.*, B, **77**, 531.  
 BÜRGERS, TH. J., 1910, *Z. ImmunForsch.*, Tl. I, Orig., **5**, 638.  
 BÜRGERS, TH. J. & MEISNER, W., 1911, *Z. ImmunForsch.*, Tl. I, Orig., **11**, 528.  
 CAULFIELD, A. H., 1908, *J. Infect. Dis.*, **5**, 245.  
 CLARK, C. P. & SIMONDS, J. P., 1908, *J. Infect. Dis.*, **5**, 1.  
 COMANDON, J., 1919, *C.R. Soc. Biol.*, Paris, **82**, 1171, 1305.  
 COWIE, D. M. & CHAPIN, W. S., 1907-8, *J. Med. Res.*, **17**, 95.  
 DEAN, G., 1905, *Proc. Roy. Soc.*, B, **76**, 506; 1907<sup>1</sup>, *ibid.*, B, **79**, 399; 1907<sup>2</sup>, *Brit. Med. J.*, ii, 1409.  
 DENYS, J., 1898, *Zbl. Bakter.*, Abt. I, **24**, 685.  
 DENYS, J. & LECLEF, J., 1895, *Cellule*, **11**, 177.  
 DUDGEON, L. S., 1908, *Proc. Roy. Soc.*, B, **80**, 531.  
 v. DUNGERN, FR., 1900, *Münch. med. Wschr.*, **47**, 677, 962.  
 EGGERS, H. E., 1908, *J. Infect. Dis.*, **5**, 263.  
 FENN, W. O., 1921-2, *J. Gen. Physiol.*, **4**, 373.  
 FLEMING, A., 1927, *Brit. J. Exp. Path.*, **8**, 50.  
 FLEXNER, S. & JOBLING, J. W., 1908, *J. Exp. Med.*, **10**, 141.  
 FORNET, W., 1909, *Zbl. Bakter.*, Abt. I, Ref., **44**, Beih. 20.\*  
 FORNET, W. & PORTER, A. E., 1908-9, *Zbl. Bakter.*, Abt. I, Orig., **48**, 461.  
 FRIEDBERGER, E. & HARTOCH, O., 1910, *Z. ImmunForsch.*, Tl. I, Orig., **4**, 218.  
 FRIEDBERGER, E. & MORESHI, C., 1908, *Zbl. Bakter.*, Abt. I, Orig., **45**, 346.  
 GLYNN, E. E. & COX, G. L., 1909-10, *J. Path. Bact.*, **14**, 90.  
 GORDON, J., WHITFHEAD, H. R. & WORMALL, A., 1926, *Biochem. J.*, **20**, 1028, 1044; 1929, *J. Path. Bact.*, **32**, 57.  
 v. GRUBER, M., 1909, *Zbl. Bakter.*, Abt. I, Ref., **44**, Beih. 2.\*  
 v. GRUBER, M. & FUTAKI, K., 1906, *Münch. med. Wschr.*, **53**, 249; 1907, *ibid.*, **54**, 249.  
 HAENDEL, 1908, *Arb. GesundhAmt.*, Berl., **28**, 523.  
 HATA, S., 1908, *Z. Hyg. InfektKr.*, **61**, 81.  
 HEKTOEN, L., 1906, *J. Infect. Dis.*, **3**, 102; 1908, *ibid.*, **5**, 249; 1909<sup>1</sup>, *ibid.*, **6**, 66; 1909<sup>2</sup>, *ibid.*, **6**, 78; 1911, *J. Amer. Med. Ass.*, **57**, 1579.  
 HEKTOEN, L. & CARLSON, A. J., 1910, *J. Infect. Dis.*, **7**, 319.  
 HEKTOEN, L. & TUNNICLIFF, R., 1921, *J. Infect. Dis.*, **29**, 553.  
 HUGGENBERG, E., 1910, *Zbl. Bakter.*, Abt. I, Orig., **55**, 53.  
 JOBLING, J. W., 1909, *J. Exp. Med.*, **11**, 614.  
 KEITH, R. D., 1906, *Proc. Roy. Soc.*, B, **77**, 537.  
 KITE, G. L. & WHERRY, W. B., 1915, *J. Infect. Dis.*, **16**, 109.  
 KLIEN, H., 1907, *Johns Hopk. Hosp. Bull.*, **18**, 245.  
 KRAUS, R. & ST. BAECHER, 1909, *Z. ImmunForsch.*, Tl. I, Orig., **3**, 9.  
 LAMBOTTE, U. & STIENNEN, T., 1906, *Zbl. Bakter.*, Abt. I, Orig., **40**, 224, 393, 503.  
 LEDINGHAM, J. C. G., 1907, *Proc. Roy. Soc.*, B, **79**, 482; 1908, *ibid.*, B, **80**, 188; 1909, *Z. ImmunForsch.*, Tl. I, Orig., **3**, 119; 1912, *J. Hyg., Camb.*, **12**, 320.  
 LEDINGHAM, J. C. G. & DEAN, H. R., 1912, *J. Hyg., Camb.*, **12**, 152.  
 LEISHMAN, W. B., 1902, *Brit. Med. J.*, i, 73; 1905, *Trans. Path. Soc., Lond.*, **56**, 344.  
 LEISHMAN, W. B., HARRISON, W. S., SMALLMAN, A. B. & TULLOCH, F. M. G., 1905, *J. Hyg., Camb.*, **5**, 380.  
 LEVADITI, C. & INMAN, 1907, *C.R. Soc. Biol.*, Paris, **62**, 683, 725, 817, 869.  
 LEVADITI, C. & KÆSSLER, K. K., 1907, *C.R. Soc. Biol.*, Paris, **62**, 685.  
 LEVADITI, C. & MUTERMILCH, ST., 1910, *C.R. Soc. Biol.*, Paris, **68**, 1079.  
 LEVADITI, C. & ROCHE, J., 1907, *C.R. Soc. Biol.*, Paris, **62**, 619.  
 LINDEMANN, 1910-11, *Arb. GesundhAmt.*, Berl., **38**, 163.  
 LÖHLEIN, M., 1905, *Ann. Inst. Pasteur*, **19**, 647; 1906<sup>1</sup>, *ibid.*, **20**, 939; 1906<sup>2</sup>, *Zbl. Bakter.*, Abt. I, Ref., **38**, Beih. 32\*; 1907, *Münch. med. Wschr.*, **54**, 1473.  
 MACDONALD, G. G., 1906, *Studies in Pathology*, Aberdeen University, 365.  
 MADSEN, TH. & WULFF, O., 1919, *Ann. Inst. Pasteur*, **38**, 437.

MARCHAND, L., 1898, *Arch. méd. exp.*, **10**, 253.  
 MARKL, G., 1908, *Z. Hyg. InfektKr.*, **42**, 244.  
 MARSHALL, W. E., 1907, *J. Path. Bact.*, **12**, 378.  
 MENNES, FR., 1897, *Z. Hyg. InfektKr.*, **25**, 413.  
 MUDD, S. & MUDD, E. B. H., 1926, *J. Exp. Med.*, **43**, 127 ; 1927, *ibid.*, **46**, 173.  
 MUDD, S., LUCKÉ, B., McCUTCHEON, M. & STRUMIA, M., 1929, *J. Exp. Med.*, **49**, 779, 797.  
 MUIR, R. & MARTIN, W. B. M., 1906, *Brit. Med. J.*, ii, 1783 ; 1907, *Proc. Roy. Soc.*, B, **79**, 187.  
 NEUFELD, F., 1908<sup>1</sup>, *Arb. GesundhAmt.*, Berl., **27**, 414 ; 1908<sup>2</sup>, *ibid.*, **28**, 125 ; 1910,  
*ibid.*, **33**, 580 ; 1913, *Handb. d. path. Mikroorg.*, hrsg. Kolle und Wassermann,  
Jena, **2**, (1), 401.  
 NEUFELD, F. & BICKEL, 1908, *Arb. GesundhAmt.*, Berl., **27**, 310.  
 NEUFELD, F. & HAENDEL, 1908<sup>1</sup>, *Arb. GesundhAmt.*, Berl., **28**, 198 ; 1908<sup>2</sup>, *ibid.*,  
**28**, 572.  
 NEUFELD, F. & HÜNE, 1907, *Arb. GesundhAmt.*, Berl., **25**, 164.  
 NEUFELD, F. & RIMPAU, W., 1904, *Deuts. med. Wschr.*, **30**, 1458 ; 1905, *Z. Hyg. InfektKr.*, **51**, 283.  
 NEUFELD, F. & TÖPFER, H., 1905, *Zbl. Bakt.*, Abt. I, Orig., **38**, 456.  
 NEUMANN, R. O., 1907, *Zbl. Bakt.*, Abt. I, Orig., **44**, 46.  
 NOGUCHI, H., 1907, *J. Exp. Med.*, **9**, 455.  
 OHKUBO, S., 1910, *Z. ImmunForsch.*, Tl. I, Orig., **4**, 1.  
 OPIE, E. L., 1907, *J. Exp. Med.*, **9**, 515.  
 v. PHILIPSBORN, E., 1924, *Deuts. Arch. klin. Med.*, **145**, 351.  
 PONDER, E., 1925-6, *J. Gen. Physiol.*, **9**, 827.  
 PORGES, O., 1909, *Z. ImmunForsch.*, Tl. I, Orig., **2**, 4.  
 RHUMBLER, L., 1910, *Arch. EntMech. Org.*, **30**, 194.  
 ROSENOW, E. C., 1906, *J. Infect. Dis.*, **3**, 683 ; 1907, *ibid.*, **4**, 285 ; 1910, *ibid.*, **7**, 429.  
 ROSENTHAL, W., 1909<sup>1</sup>, *Zbl. Bakt.*, Abt. I, Ref., **42**, Beih. 177\* ; 1909<sup>2</sup>, *ibid.*,  
Abt. I, Ref., **44**, Beih. 14\*.  
 RUEDIGER, G. F., 1905, *J. Amer. Med. Ass.*, **44**, 198 ; 1906<sup>1</sup>, *ibid.*, **46**, 108 ; 1906<sup>2</sup>,  
*J. Infect. Dis.*, **3**, 156.  
 RUEDIGER, G. F. & DAVIS, D. J., 1907, *J. Infect. Dis.*, **4**, 333.  
 RUSSELL, F. F., 1907, *Johns Hopk. Hosp. Bull.*, **18**, 252.  
 SACHS, H. & TERUUCHI, Y., 1907, *Berl. klin. Wschr.*, **44**, 467.  
 SAWTCHENKO, J.-G., 1902, *Ann. Inst. Pasteur*, **16**, 106.  
 SCHNEIDER, 1910-11, *Zbl. Bakt.*, Abt. I, Ref., **48**, 271.  
 SCHÜTZE, H., 1914, *J. Hyg., Camb.*, **14**, 201.  
 SHATTOCK, S. G. & DUDGEON, L. S., 1908, *Proc. Roy. Soc.*, B, **80**, 165.  
 SIA, R. H. P., 1928, *Zbl. Bakt.*, Abt. I, Ref., **88**, 340.  
 SIMON, C. E., LAMAR, R. V. & BISPHAM, W. N., 1906, *J. Exp. Med.*, **8**, 651.  
 SMITH, J. H. & BROOKS, R. ST. J., 1912, *J. Hyg., Camb.*, **12**, 77.  
 TCHISTOVITCH, N. & YOUREVITCH, V., 1908, *Ann. Inst. Pasteur*, **22**, 611.  
 THOMSEN, O. & WULFF, F., 1920, *C.R. Soc. Biol.*, Paris, **83**, 943.  
 TOBLER, W., 1924, *Z. ges. exp. Med.*, **41**, 558.  
 TUNNICLIFF, R., 1908, *J. Infect. Dis.*, **5**, 14 ; 1910, *ibid.*, **7**, 698.  
 UNGERMANN, E., 1910, *Z. ImmunForsch.*, Tl. I, Orig., **5**, 269.  
 WENT, S., 1923, *Z. ImmunForsch.*, Tl. I, Orig., **37**, 408.  
 WRIGHT, A. E., 1926, *Lancet*, Lond., i, 4.  
 WRIGHT, A. E. & DOUGLAS, S. R., 1903-4, *Proc. Roy. Soc.*, **72**, 357 ; 1904, *ibid.*, **73**,  
128 ; 1904-5, *ibid.*, **74**, 147, 159.  
 WRIGHT, A. E. & REID, S. T., 1906, *Proc. Roy. Soc.*, B, **77**, 211.  
 ZADE, M., 1909, *Z. ImmunForsch.*, Tl. I, Orig., **2**, 81.  
 ZINSSER, H. & CAREY, E. G., 1914, *J. Exp. Med.*, **19**, 345.

## CHAPTER XII. AGGLUTINATION.

By J. A. ARKWRIGHT (LISTER INSTITUTE).

### Introduction.

ALTHOUGH the aggregation of bacteria growing in the serum of an immune animal was observed by Charrin and Roger (1889), the specific agglutination reaction as now understood was first described by Gruber and Durham (1896). It was at once recognized by Grunbaum (1896), Widal (1896<sup>1</sup>), and Widal and Sicard (1897) that agglutination was of great value for diagnostic purposes in typhoid fever, since the agglutinating antibodies or agglutinins appeared in the serum of the infected patient during the first 7 to 10 days of the illness and a few drops of blood were sufficient for a test with a culture of bacteria. The reaction was first practised in typhoid fever, but its use was soon extended to other diseases, though often with indifferent success. The ease with which the test was made and its consequent popularity were largely dependent, as we now know, on the special suitability of flagellated bacilli and particularly of *B. typhosus* for the reaction. Agglutination was also found very valuable for determining the nature of unknown bacteria with the sera of animals which had been inoculated with known cultures. An immense amount of work was done in the next few years to determine the conditions for the formation of agglutinins in the serum of patients and in artificially immunized animals. The results obtained were found in many respects to be applicable to other antibodies.

Specific agglutination, as was shown by Bordet (1899), occurs in two stages. The first is the preparation or sensitization of the bacteria by a specific substance (agglutinin) contained in the serum of immune animals. The sensitization can only be recognized by the subsequent behaviour of the bacteria. The agglutinins confer on the bacteria the property of clumping in the presence of neutral salts in low concentration. The second stage consists of the agglomeration of the sensitized bacteria into clumps in the presence of electrolytes; and the clumps are then deposited by gravity. The two stages usually proceed simultaneously though the second stage requires a much longer time than the former. By keeping the mixture of bacteria and diluted serum as free as possible from electrolytes the second stage can be postponed almost indefinitely until salt is added.

The latter stage, during which agglutination actually takes place, must be regarded as a special instance of the physical phenomenon of aggregation and precipitation of very small particles of different kinds in suspension in water, as the result of the addition of electrolytes. Familiar

instances of this influence of electrolytes are the well-known instability of colloidal solutions of metals in the presence of very low concentrations of salts and acids, and the flocculation of finely divided clay or other substances suspended in water when electrolytes, especially the salts of calcium and magnesium, are added, as happens when muddy river water enters the sea.

### Agglutination of Bacteria : Specific and Non-Specific.

The conditions governing this process belong to the realm of colloidal physics and are very imperfectly understood (see Vol. I, Chapter III ; and Vol. VI, Chapter XVI). However, a number of facts concerning the behaviour of suspensions of bacteria have been experimentally ascertained (Porges, 1905; Porges and Prantschoff, 1906; Arkwright, 1914<sup>1 & 2</sup>; Northrop, 1928). Many bacterial suspensions are agglutinated without previous sensitization by agglutinins, but the agglutination of sensitized and of unsensitized bacteria appear to be controlled by similar physical conditions. A large number of factors are, no doubt, involved in this process, but two main sets of forces must be recognized ; those tending to make the particles cohere when they come into contact and the forces which keep them apart in a stable suspension. In addition, a definite attractive force between the particles bringing them together has been assumed to be active in some cases. Under certain circumstances agglutination is due to the formation outside the bacterial bodies of a coagulum or precipitate which encloses them or otherwise induces their cohesion and so aggregates them. This form of clumping will be considered later (Paltauf, 1897 ; Kraus, 1897<sup>2</sup> ; Defalle, 1902).

### SURFACE CHARGE OR DIFFERENCE OF POTENTIAL (P.D.) BETWEEN THE PARTICLES AND THE SUSPENDING LIQUID.

Bacteria, like very many other particles suspended in water or weak salt solution, carry a negative charge on their surfaces, which keeps them dispersed by the repellent action of their similar charges. The strength of the charge varies very much with the kind of bacteria and the conditions. The positively charged cations of sodium or potassium chloride, &c., and the much more strongly charged bivalent cations of calcium and magnesium salts or the hydrogen ions of acids are believed so to influence the ionization of the amphoteric colloid on the bacterial surfaces as to bring about the observed diminution of their negative charge and the P.D.

It has been shown by Northrop and De Kruif (1921-3) that agglutination of bacteria does not occur unless the negative charge has been reduced to 15 millivolts or less. This has been confirmed by Shibley (1924, 1926), and was shown to be true both of unsensitized and specifically sensitized bacteria. Falk, Jacobson and Gussin (1925) and Falk (1928) showed that the stability of many emulsions of bacteria, e.g. S forms of pneumococci, was associated with a high negative charge (P.D.), and that spontaneously agglutinable R bacteria, on the other hand, had a low P.D.

That in a solution of salts it is the cation which is the predominant factor was shown by Bechold (1904) and Porges and Prantschoff (1906) by the use of salts and acids with the same cation (Na, K, Ca, Mg, &c.) in equal concentration but with varying anions, and of solutions of different acids of the same hydrogen-ion concentration (Beniasch, 1912). In suspensions of particles whose surface charge is near neutrality some may be positively and some negatively charged, and this state of things may actually bring about their approach by mutual attraction. In the case of unsensitized bacteria there is no evidence that this factor is ever operative and a positive charge has not certainly been demonstrated on such bacteria, at any rate, in solutions of monovalent salts.

*Cohesion.* The efficient cohesive force which holds the agglutinated bacteria together when they have come into contact has not been certainly determined and may not always be the same. If the surface energy at the bacteria-water interface is greater than that between the bacteria, then there is a tendency for them to cohere if they come in contact. The effect of electrolytes and other substances in altering in other respects the bacterial surfaces and their tendency to cohere may also be of considerable importance. Northrop found that in the case of some unsensitized bacteria, neutral salt solution might lower the surface charge to less than 15 millivolts without agglutination occurring, but that after sensitization with agglutinins when the charge was lowered to the same extent agglutination occurred. He maintained that this showed that the agglutination was not determined by the reduction of charge by the electrolytes, but by the alteration of the surface by the agglutinins. The very low concentration of sodium chloride required to clump sensitized bacteria suggests that the actual agglutination is to some extent due to the charge being lowered by the cations more readily after sensitization. The very much lower concentration of bivalent and trivalent cations (Ca, Mg and La) than of monovalent cations needed to induce clumping is also evidence of the importance of the electric charge.

#### AGGLUTINATION OF BACTERIA BY COHESION OF THE DIFFERENT SURFACES WHICH THEY PRESENT.

Clumping of bacteria must be due either to the contact and cohesion of their surfaces, or to the coagulation or precipitation of some interbacillary substance present in the suspension. Different kinds of agglutination must be recognized which are due to the cohesion of the different parts of the bacteria.

The surfaces of different parts of bacteria are by no means uniform.

*Flagella* present very large surfaces for agglutination, and these cohere and form clumps under different conditions to the bacterial bodies. Such an agglutination of flagella may give the appearance of coagulation of an interbacillary substance.

The *bodies* of bacteria may agglutinate by cohesion of their surfaces quite independently of the flagella. This somatic agglutination may occur

whether the flagella are agglutinated or not. In the former case the clumped flagella may separate the bodies for a time, and in the latter case, the bodies may be in contact and coherent whilst the flagella are still free and actively motile.

In the agglutination of capsulated bacteria the clumping must be due to the cohesion of the *capsules* without the actual contact of the surfaces of the bodies which are surrounded by the capsule, but this process has been incompletely studied and only in sensitized bacteria.

#### COAGULATION OF INTERBACILLARY SUBSTANCE.

In some other cases, especially in old cultures, dissolved or detached portions of the bacteria may be present in the liquid and form a *precipitate* which, under special conditions, entangles the bacterial bodies, e.g. emulsions of old cultures of dysentery bacilli.

The character of the clumping when the flagella agglutinate is generally quite different from that seen in somatic agglutination. The flagellar clumps appear much more quickly and are large, loose and fluffy, being formed by the cohesion and interlacing of flagella, whether these are attached to bacteria or are free in the suspension. These clumps are easily broken up by shaking. The clumps formed when the bodies cohere are very much smaller and denser and appear as small granules which may be the size of grains of sand or too small to be seen by the naked eye. The clumps cohere much more firmly and are not nearly so readily affected by shaking as the flagellar clumps.

#### Non-Specific Agglutination of Bacteria in the Absence of Specific Sera.

#### THE EFFECT OF NEUTRAL SALTS ON SOMATIC AGGLUTINATION IN THE ABSENCE OF AGGLUTININS.

Many bacteria agglutinate spontaneously, i.e. without preliminary sensitization, in the presence of weak salt solutions and some even in distilled water. This phenomenon appears to be confined to the bodies of the bacteria and not to affect the flagella (White, 1925, 1926; Arkwright, 1927<sup>3</sup>). The most potent salts are those with bivalent or trivalent cations (Porges and Prantschoff, 1906). The rough or R form of the Gram-negative group of intestinal bacteria is characterized by its susceptibility to agglutination by salts. Dilutions of sodium chloride are usually effective in a concentration of 0·85 per cent. (N/7). Calcium chloride N/64 or lanthanum nitrate N/1600 will agglutinate the same suspensions (Arkwright, 1914). Such R suspensions are generally stable in 0·1 per cent. sodium chloride and often in 0·4 per cent. They may be more stable in 2·8 per cent. NaCl than in 0·85 per cent. Suspensions of the smooth or S form of the same strain are quite stable in sodium chloride 1·4 per cent. or weaker solutions (Arkwright, 1914<sup>1</sup>, 1921).

Removing the flagella by shaking and washing the bacteria has no effect on the salt agglutinability, and in motile and non-motile variants

the R character and salt agglutinability may be equally well marked. In broth cultures and suspensions in salt solution containing living motile R bacteria the bodies may be seen to be in contact, whilst the clumps are carried about by the flagella, which are unclumped by the salt and are still active (Arkwright, 1927<sup>3</sup>). The clumps of R cultures formed in salt solution are usually very small and often microscopic, but may be coarsely granular. They are fairly coherent and persistent when shaken.

Heating stable suspensions of S cultures at 100° C. for half to two hours does not make them agglutinable by salt, but heating R suspensions at 100° C. often increases their tendency to spontaneous agglutinability (White, 1926, 1928).

It has been shown by White (1926, 1928) that agglutination of R suspensions of *Salmonella* in salt solution does not occur if the bacteria have been previously extracted by alcohol and chloroform; a procedure which removes some substance which White regards as a lipoid. This 'lipoid' is, however, not peculiar to the R variant, but is also present in the S form. It seems, therefore, that the stability of the S bacteria in suspensions is probably due to the presence of something which neutralizes the clumping tendency of the 'lipoid'. It is reasonable to suppose, as White suggests, that the substance which makes for stability is identical with the 'specific soluble substance' (carbohydrate) which is present in the S form and determines the serological differences between S and R cultures (Heidelberger and Avery, 1923; Zinsser and Parker, 1923; Furth and Landsteiner, 1928; White, 1926).

In some large groups and species of bacteria most of the members have the character of spontaneous agglutinability in weak salt solutions or even in distilled water, so that it is very difficult to obtain a stable uniform suspension of the bacteria. However, certain strains can generally be found even in these groups which can be satisfactorily emulsified. Felix finds that suspensions of some strains of *B. proteus* and anaerobic bacteria are more stable in 0·85 per cent. NaCl than in weaker solutions or distilled water (personal communication). Amongst those organisms which make very unstable suspensions may be mentioned *Staphylococcus*, *Streptococcus*, many *Corynebacteria*, *B. tuberculosis*, *M. catarrhalis*, &c.

#### *Relation of P.D. to Non-Specific Agglutination by Salts.*

In some of the last-mentioned bacteria it has been shown (Northrop, and de Kruif 1921-2; Shibley, 1925; Falk, 1928) that the surface charge (P.D.) is very low and it is believed that the cohesive force is often unusually great. The amount of surface charge may vary in closely allied strains, and Falk, Jensen and Mills (1927) claim that it is possible to distinguish toxigenic from non-toxigenic strains of *C. diphtheriae* by the lower P.D. carried by the former. This observation needs confirmation, but it is in accordance with the well-known spontaneous agglutinability of very many strains of 'virulent' *C. diphtheriae*, and the ease with which stable suspensions can often be made from some 'non-virulent' strains of the

same organism and also from cultures of the allied non-pathogenic *C. hofmanni*. It is not the case, however, that all strains of toxigenic *C. diphtheriae* are spontaneously agglutinable.

*The Effect of Certain Additions to Suspensions on their Agglutinability by Salt.*

It has been shown by Loeb (1922-4) that if a dilute solution of egg albumin is added to a suspension of particles of collodion, they become agglutinable by sodium chloride, and the same has been found true in the case of bacteria (Shibley, 1926). It has been suggested in explanation that the albumin forms an extremely thin coating on the bacteria or other particles and in this form acts like denatured albumin, which is known to be agglutinated by salt.

The bodies of bacteria are sometimes agglutinated by normal serum even in the absence of 'normal agglutinins'. White (1928) has pointed out certain conditions under which spontaneous agglutination occurs. He describes four forms of non-specific agglutination : (1) By electrolytes (salts) determined by the bacterial 'lipoid' and following loss of specific soluble substance ; the salt agglutinability of R variants. (2) By electrolytes and due to unusual intensity of the influence of bacterial 'lipoid', without loss of specific soluble substance. (3) By electrolytes following denaturation of the bacterial protein in the absence of specific soluble substance ; the specially increased salt agglutinability of heated (100° C.) R variants. (4) In the presence of serum and due to a reaction of certain 'lipoid' constituents of the bacteria with the serum ; the serum agglutination by normal serum of certain R variants. The last, he states, may occur in a high dilution of normal serum (1 in 500 to 1 in 1,000).

**AGGLUTINATION BY ACID.**

It was shown by Michaelis (1911) that *B. typhosus* was agglutinated in the presence of acid at a definite hydrogen-ion concentration which was characteristic of the species, and that certain allied bacteria such as *B. paratyphosus* and *B. enteritidis* (Gaertner) were agglutinated at slightly different pH. A number of buffer salt solutions of varying hydrogen-ion concentrations from  $1.8 \times 10^{-5}$  to  $10^{-3}$  were prepared, usually with acetic acid or lactic acid and sodium hydrate. The different solutions ascended in the series of hydrogen-ion concentrations in multiples of 2. To 1.1 c.cm. of each of these solutions was added 0.5 c.cm. of an emulsion of the bacteria in distilled water. The mixtures were incubated for 10 to 20 minutes at 37° C., and the pH of the tube in which flocculation first occurred was noted and taken as the optimum pH characteristic for the strain of bacteria tested.

*Flagellar Agglutination.*

Whereas most strains of *B. typhosus* agglutinate first at  $3.5 \times 10^{-5}$  or  $0.7 \times 10^{-4}$  and never in weaker acid, *B. paratyphosus A* and *B* both require a higher acidity, about  $2.5 \times 10^{-4}$ . The clumps formed are very

loose and fluffy but soon settle down in the tube. After standing, agglutination also appears in 3 or 4 more tubes towards the acid end of the series. There is little doubt that the agglutination at about  $10^{-5}$  to  $10^{-4}$  is due to the flagella. This is the reason for the fact noted by Beniasch (1912), Beintker (1912) and others, that about 10 per cent. of *B. typhosus* strains examined do not agglutinate in this zone of acidity, since strains which are non-motile or have feebly developed flagella do not clump at this pH, i.e. 5 to 4. The correspondence between agglutinability by serum and by acid was found to be fairly close though the specific serum was a more delicate test.

#### *Somatic Agglutination by Acid.*

It was pointed out by Arkwright (1914<sup>1 & 2</sup>) that agglutination, but of a more granular kind, also occurred at about pH 3·0, and that this latter zone of agglutination was about the same for many kinds of bacteria, including *B. typhosus*, *B. paratyphosus*, *B. coli*, *B. dysenteriae*, *Staphylococcus*, &c. If the bacteria in an emulsion of *B. typhosus* are thoroughly washed free from flagella it is found that they are only agglutinable at about pH 3·0 and no longer in the less acid zone described as characteristic by Michaelis. An emulsion of a non-motile strain also behaves in the same way as bacteria deflagellated by shaking and washing. This agglutination of the bodies at about pH 3·0 may, therefore, be called *somatic* agglutination by acid.

#### *Effect of Heating the Suspension.*

It was shown by Beniasch (1912) that if an emulsion of *B. typhosus* or *paratyphosus* was heated at 100° C. for half an hour, it no longer agglutinated at the characteristic pH ( $[H^-] = 3\cdot5 \times 10^{-5}$ ), but only at about pH 3·0. This was confirmed by Arkwright (1914<sup>1 & 2</sup>), who found that the zone of pH for clumping the washed unheated bacterial bodies and the boiled bacteria was approximately the same. The occurrence of distinct somatic and flagellar agglutination at different zones of pH appears to be established and the relative stability and lability, when heated, of the two agglutinable substances concerned are the same for acid and for serum agglutination of *B. typhosus* (Arkwright, 1928) and *B. proteus* (Putter and Zorn, 1924).

It was shown by Arkwright (1914<sup>1</sup>) that if a flagellated suspension of *B. typhosus* had been heated at 80° C. for 30 minutes, both kinds of acid agglutination (flagellar and somatic) were inhibited, a phenomenon which also occurs in the case of agglutination with specific serum (Porges and Prantschoff, 1906).

*Emulsions of separated flagella.* Not only is a suspension of whole bacteria liable to flagellar agglutination by acid, but in a clear centrifuged watery extract of flagellated bacteria a flocculent precipitate can be obtained at the same pH. This, no doubt, is due to clumping of the flagella; if the extract has been heated at 100° C., this clumping no longer occurs.

Observations on the acid agglutination of spontaneously agglutinable bacteria cannot be carried out very satisfactorily by this technique, since in these strains the agglutination of the bodies by the acid and salts occurs over too wide a range of pH and obscures the characteristic zone of clumping.

#### *Cataphoresis.*

Northrop (1928), Beniasch (1912), Arkwright (1914), Northrop and de Kruif (1921-3), could not demonstrate by cataphoresis experiments that the agglutination by acid took place at the isoelectric point, with a reversal of charge on the bacteria at a pH of higher acidity, such as occurs in the case of globulin, &c. (Rona and Michaelis, 1910; Michaelis, 1911; Chick and Martin, 1912). Since the acid agglutination which Michaelis was observing is now believed to be due to the flagella and since the bodies, which have a different zone of pH for agglutination, are also present in emulsions of *B. typhosus* and are the chief part of the bacteria, perhaps it is hardly to be expected that cataphoresis would give the result which Michaelis anticipated. Beniasch was, therefore, correct in attributing this failure to the probable existence of a mixture of substances with different isoelectric points in the emulsion. The movement in cataphoresis of flagella cannot readily be observed microscopically, and this has hindered direct observations on the charge on the flagella and bodies independently.

With regard to somatic agglutination an isoelectric point for the bodies has not been satisfactorily demonstrated at the pH at which they are agglutinated, though reduction of surface charge has often been shown.

#### *Effect on Acid Agglutination of Additions to Bacterial Emulsions.*

If a clear centrifuged watery extract of motile *B. typhosus*, which consists largely of an emulsion of flagella, or if a suspension of whole flagellated *B. typhosus* is mixed with a suspension of bacteria of another kind, e.g. *B. coli*, or even with indifferent particles such as mastic or kieselguhr, a flagellar precipitate can be procured by adding an acid agglutinating buffer mixture of suitable pH, and the clumps of flagella include and carry down the added bacteria or other particles. If a little normal serum, e.g. 1 in 1,000 horse-serum, is added to a suspension of bacteria, clumping and precipitation is then obtained at a pH approximately the same as that at which globulin would precipitate, and in this case the zone of agglutination is narrower than when only flagellated bacteria are present, and no agglutination occurs at pH 3·0, the zone of agglutination of the untreated bodies. It can be shown by cataphoresis experiments that the bacteria are precipitated at the isoelectric point of the globulin, and that they carry a negative charge on the alkaline side and a positive charge on the acid side of this pH, and, in fact, behave in this respect like particles of globulin with which they are presumably wholly or in part coated.

**Specific Serum Agglutination.****AGGLUTININS.**

The substances contained in the blood-serum of an immune animal which cause agglutination are known as agglutinins, and are examples of the so-called 'antibodies' which result from infection with microbes or from the parenteral introduction into the animal body of substances capable of eliciting immunity reactions with changes in the blood.

According to the hypothesis of Ehrlich the agglutinins were chemically characterized receptors set free into the blood-stream as the result of their excessive reproduction due to the stimulation of the receptors in the animal cells by bacteria introduced into the animal body and acting as antigens. In this view agglutinins consisted essentially of two chemical receptors, a haptophore group which combined with the antigen and an ergophore which effected the clumping. This hypothesis had to be abandoned as a complete explanation of the facts, when it became clear, as the result of the work initiated by Bordet, that complex physical changes in the states of colloidal aggregation were essential factors, both in the union of antigen and agglutinin and in the actual clumping of the agglutinin-antigen complex. The chemical nature of the specific affinity of the agglutinin for the antigen remains as the generally accepted hypothesis.

The exact composition and the chemical and physical nature of agglutinins are unknown, since they have never been obtained free from adventitious substances. Felton (1928) has obtained antibodies to the pneumococcus freed from 90 per cent. of the serum protein and much of the serum lipoid. The specifically precipitable protein was found by Heidelberger and Kendall (1929) to be 39 to 47 per cent. of the total protein in the solution, calculated from the nitrogen present. The antibodies are very closely associated with the protein of the serum and in some instances with the pseudo-globulin (Schiff, 1925). Agglutinins for different parts of bacteria (bodies and flagella) are probably connected with different fractions of the serum proteins, since they are differently affected by heat. The agglutinins correspond to the particular parts of the bacterium which have provoked their production in the animal body. The substances which evoke them are known as agglutinogens or antigens. The peculiar property of agglutinins is that they combine with their corresponding agglutinogens to promote the agglutination and deposition of the bacteria.

The number, qualities and amount of the agglutinins in a serum can only be revealed and analysed by the use of their corresponding antigens, and the converse is also to some extent true, but it is often more difficult to separate the agglutinins for use as reagents than to obtain the different antigens separately. Whilst certain coarse differences in the agglutinable substances can be recognized by chemical or physical means, e.g. agglutination by acids and salts, the finer specific differences as a rule can only be revealed by the production of specific antibodies and reactions with them *in vitro*.

## AGGLUTINOGENS, ANTIGENS AND AGGLUTINABLE SUBSTANCES.

It is customary to speak of the agglutinable substances as agglutinogens or antigens because there is reason to believe that in the majority of cases the substance which excites the formation of agglutinins in the animal body, i.e. the agglutinogen, is the same as that which is agglutinated *in vitro*. From the presence of a given agglutinable substance in a bacterial emulsion it is usually possible to foretell the appearance of a corresponding agglutinin in the serum of the animal inoculated. In the case of precipitation of special proteins by immune bodies in serum the identity of the precipitable substance and precipitinogen is more easily demonstrated, since in the case of the precipitin reaction the antigen is of relatively simple composition. Whilst it is probably always true that agglutinable bacteria produce agglutinins when inoculated, there are exceptions to the rule that the precipitable substances derived from bacteria are always antigenic, i.e. capable of eliciting antibodies *in vivo*. As an instance may be given the 'specific soluble substance' derived from the capsule of the pneumococcus, which is precipitable *in vitro* with specific antipneumococcus serum but not able to evoke antibodies *in vivo*.

On the other hand, some bacterial emulsions which are antigenic are not themselves agglutinated by the serum agglutinins which they evoke. For instance, cultures of *B. typhosus* have been isolated from the blood in typhoid fever which were found not to be agglutinated with a highly agglutinating rabbit's serum or with the serum of the patient which clumped a stock culture of the same micro-organism. Nevertheless, the inagglutinable strain when inoculated into a fresh animal stimulated the production of agglutinins for a normal strain. Similar instances have often been observed. The inagglutinable strains are said to be serum-fast and are supposed to have become resistant to the patient's serum by residence in the body, suggesting an adaptation by survival of the fittest individual bacilli.

This anomalous phenomenon was explained on Ehrlich's hypothesis that the antigen contained two chemical groups, the one, 'haptophore', which joined with the cells in the animal body and stimulated the production of antibodies and also united with the agglutinin *in vitro*, the other, 'ergophore', which actually brought about clumping of the bacteria and was lost in special instances. In the majority of cases the observations on the inagglutinability of bacterial antigens are explicable on the ground that the antigenic substance which is chiefly concerned in agglutination is only present in very small quantities, which are insufficient to produce visible agglutination, but are capable of stimulating the formation of a moderate amount of antibodies if the inoculum is large enough. Such inagglutinable strains of *B. typhosus* are often if not always due to a very poor development of the flagellar antigen. It is unnecessary in most cases to assume modified agglutinogen, which is antigenic but not agglutinable, as in the case of modified toxin or toxoid, which is antigenic but not toxic.

## THE MECHANISM OF AGGLUTINATION.

The mechanism of agglutination was early studied by Pfeiffer (1896). Gruber (1899), Paltauf (1897) and Bordet (1899) and others. Craw (1905) summarized the various divergent views and adopted that of Bordet, which is now generally accepted. Craw made experiments on the fixation of specific agglutinins by bacteria, and put forward a formula for the physical phenomena involved, which he showed were very similar to those of adsorption of dyes. He opposed the view of Arrhenius (1903) based on the experiments of Eisenberg and Volk (1902) that the agglutinins were fractionally distributed between the bacilli and the medium according to the laws of diffusion.

A peculiar effect of some agglutinating sera known as the zone phenomenon has been extensively investigated. It is especially noticed with sera which have been kept some time or heated at 60 to 70° C. It consists in the absence of clumping in strong serum, e.g. 1 in 50 to 1 in 100, but its appearance in further dilutions up to a high titre.

Eisenberg and Volk (1902) made a large number of experiments with *B. typhosus* with a view to determining the nature of the union of antigen and antibody and the resulting agglutination. They examined the effect of heat and chemical substances on suspensions of bacteria (65 to 120° C.) and on agglutinating sera (60 to 75° C.). They tested these reagents both before and after treatment, by direct agglutination experiments and by absorbing the agglutinins from diluted serum with varying doses of bacteria and then testing the amount of agglutinin left in the medium. Their main conclusion was that heat and other agencies destroyed the agglutinophore and left the haptophore groups relatively unaltered. The altered agglutinins were called agglutinoids. Dreyer and Jex-Blake (1906), working with *B. coli*, also made a very large series of observations on the effect on the agglutination reaction of heating the bacterial suspensions, (*B. coli* and *B. typhosus*) and the corresponding agglutinins in horse-serum. They found great difficulty in getting results of a high degree of accuracy and regularity. In general they confirmed the results obtained by others, but obtained differences in detail. They especially studied the production of 'zone effects' with serum which had been heated at 70 to 75° C. The weakest and strongest concentrations of such serum sometimes caused agglutination, but not the intermediate dilutions. They pointed out, however, that when the agglutination tubes were again examined after 18 hours the zones had for the most part disappeared and attributed the zones to the production by heat of a substance which impeded agglutination. They confirmed Porges' (1905) observation that heating at 80° C. may destroy the agglutinability of an emulsion, which is restored by heating at 100° C. Their interpretation of the phenomena observed was different from that of Eisenberg and Volk, since they did not accept the hypothesis of the destruction of the ergophore groups and the production of agglutinoids.

These investigations as well as those of most other workers at this time were confused and the interpretations to some extent vitiated by the failure to recognize the existence of distinct flagellar and somatic agglutinins and antibodies. It has been widely accepted of recent years that the inhibition of clumping in a zone of stronger serum was due to the presence of a non-specific protective colloid which was diluted out in lower concentrations.

Shibley (1929) reinvestigated the zone phenomenon in agglutination. In most of his experiments he used dysentery bacilli and thereby eliminated errors due to the flagellar antigen and agglutinins. He studied chiefly the zone of absent clumping which occurred when agglutinating serum had been heated at temperatures between 62 and 76° C. for 6 to 10 minutes, but the exact temperature varied with different samples of serum. He came to the conclusion that the phenomenon was due to the change of agglutinin into agglutinoid, and that the latter had a greater affinity for the bacteria than the agglutinin. Agglutinoid did not promote agglutination, and, therefore, in the more concentrated serum prevented the agglutinin from uniting with the antigen, but in the higher dilutions the agglutinin alone was present in effective concentration. The agglutinoid he conceived as combining with the bacteria but not causing them to be coated with denatured globulin, a condition essential to clumping in the presence of electrolytes according to his previously enunciated hypothesis (1926). He found that the agglutinoid could be specifically absorbed, and that the addition of other non-specific heated immune serum to the unheated specific serum did not produce the zone effect in the higher concentrations. He, therefore, rejected the hypothesis that a non-specific inhibiting substance accounted for the zone. A higher temperature, e.g. 78° C., entirely destroyed the agglutinating properties of the serum. Since it is known that somatic agglutinin is usually labile when heated at about 70 to 75° C. for 30 minutes, its progressive alteration at these temperatures is *a priori* probable. Shibley's experiments show three states of immune serum induced by heat. The unheated serum gives agglutination in all concentrations to the limit of the titre. After heating at about 60 to 70° C. a zone of inhibition occurs in the strongest serum, but agglutination takes place in the higher dilutions to the limit of the titre. After further heating at about 76° C. there is no zone, but the titre is lowered, and with serum heated to 78° C. no agglutination appears. With serum heated at 76° C. it might be supposed that the inhibition zone would have become wider, and that the agglutinoid would gradually take the place of agglutinin till clumping ceased. To explain on the lines of Shibley's hypothesis the stage at which only agglutinin is in evidence, it seems necessary to postulate two kinds of agglutinins, one of which is changed into agglutinoid at 62 to 70° C., the other which remains unaltered at 76° C. These might be associated with serum proteins of different susceptibility to heat. The results of Shibley might be compared with those of Ledingham and Dean (1912) on the bacteriotropin in serum heated at 56° C. for half an

hour. They found in serum diluted 1 in 10 a zone of lowered activity which was absent when the 'midpiece of complement' (euglobulin) had been removed with  $\text{CO}_2$ . This suggested that the inhibition was caused by altered globulin, but whether this contained specific or merely non-specific substances was not discussed.

Jones (1928) obtained apparently specific inhibition of agglutination by means of anti-hog-cholera serum heated at  $75^\circ \text{ C}$ . for 20 minutes.

#### COMBINATION OF AGGLUTININ AND AGGLUTINOGEN.

The relation of an agglutinin to its antigen is often extremely specific, and it is largely on that account that this affinity is generally believed to be chemical in nature. The question has often been discussed how far the specific affinity of the two reagents might equally well be explained by modification of the surfaces and form of colloidal particles. However, the serological specificity of proteins has been shown to vary with small chemical changes in the molecule and within a single 'species' or 'group' of bacteria with the chemical composition of the bacterial components (Heidelberger and Avery, 1924). It, therefore, seems certain that in this and other instances the specific differences have a chemical basis.

Resulting from the union due to the specific affinity, further adsorption of the agglutinins by the antigen occurs, following physical laws, and to some extent determines the quantitative relations of the two bodies, as in the case of other antigen-antibody reactions.

The union of antigen and antibody takes place very rapidly and is not generally found to be much affected by temperature. According to Porges (1905) it may be complete in 5 minutes at  $37^\circ \text{ C}$ . Gates (1922) claims to have shown that the velocity of the absorption reaction is a function of the temperature at which it occurs, and the acceleration with increased temperature is of the order of chemical phenomena.

#### THE STAGE OF CLUMPING.

As has been mentioned above, the specific agglutination of sensitized bacteria does not occur except in the presence of electrolytes. Shibley (1926) came to the conclusion that agglutination under the influence of agglutinins occurred because the sensitized bacteria were coated selectively by the globulin of the antibody, which, when in the form of a film, took on the characteristics of denatured globulin. As a result the agglutination of the coated bacteria followed the laws governing the flocculation of particles of denatured protein by electrolytes. This, of course, does not explain the specific affinity of the agglutinins for the bacteria. The actual clumping of the bacteria under the influence of salt occurs much more slowly than their sensitization by agglutinins and appears to depend on the chance meeting of the individual bacteria which cohere when they come into contact. This part of the process is hastened by movement of the suspension, which is probably the chief reason why heat accelerates the clumping. It can be shown that shaking the tube containing the

reagents with a suitable rhythm and force (Nicolle, Jouan and Debains, 1919) or causing a drop of the mixture to flow backwards and forwards on a slide very much increases the rate of clumping. Centrifuging has also been shown to hasten the reaction (Gaehtgens, 1906; Gates, 1922; Fleming, 1928). If the agglutination tubes are plunged in a water-bath at 50 to 55° C. for half their length, clumping is more rapid than if they are completely immersed, because of the convection currents set up (Topley and Platts, 1918). For the same reason, clumping is often greatly accelerated during the first 5 or 10 minutes after the tubes have been removed from the incubator at 37° C. and stood on the table at the temperature of the laboratory.

#### THREAD REACTION.

An appearance seen in young cultures grown in specific immune serum diluted with broth, which is known as the thread reaction, is almost certainly due to the same causes as produce somatic or capsular agglutination, acting during the growth and division of the bacteria in the presence of agglutinins.

It was first described by Charrin and Roger (1889) in the case of *B. pyocyanus*, and has since been often observed with other bacteria, especially the pneumococcus by Issaef (1893), Washbourn (1895), and others. Pfaundler (1898) made similar observations with *B. coli* and *B. proteus*. The technique has been used for the diagnosis of the pneumococcus. The growth, instead of being in the form of cocci or short bacilli, makes long threads or chains often arranged in a tangled mass.

#### THE DIFFERENT KINDS OF AGGLUTINOGEN AND AGGLUTININ.

In suspensions of bacteria of the *typhosus-coli* group three principal kinds of agglutinogen are to be found which determine their agglutination by serum. They correspond to those components of the bacteria described above as agglutinable by electrolytes without the aid of agglutinins. These are (1) the smooth or S somatic, (2) the rough or R somatic and (3) the flagellar or H antigen. The occurrence of flagellar agglutination of *B. typhosus* was first described by Malvoz (1897) and his view was expanded by Dineur (1898) who maintained that bacteria washed free from flagella were inagglutinable. C. Nicolle and Trenel (1902) found a very close parallelism between motility and agglutinability in cultures of *B. typhosus*, and Defalle (1902) maintained that flagella sometimes took an important part in agglutination, and also that in other cases a capsule or coating surrounding bacteria or spores was the part concerned in specific agglutination. Smith and Reagh (1903) were the first clearly to describe specific somatic and flagellar agglutination as two distinct processes and to show that the same bacteria contained both agglutinable substances, which produced in animals two corresponding agglutinins. They made their observations on motile and non-motile strains of hog-cholera bacillus which appeared to be identical except in the presence and absence respectively of motility.

They observed the now well-recognized difference between the somatic clumps, which they said were small, dense and slowly formed, and the large, loose, fluffy, flocculent clumps of flagellated bacteria, which appeared much more rapidly. The somatic agglutination which they described only occurred in a dilution of specific serum of about 1 in 200, whereas the flagellar agglutination was obtained in a dilution of 1 in 20,000. In the very small clumps resulting from the action of the somatic agglutinins the bacteria could be seen microscopically to be in close contact, even when the emulsion acted on was flagellated. Such clumps, when the bacteria were motile, were moved about by the free unagglutinated flagella. In the large loose clumps formed by agglutination of the motile strain with its corresponding serum the bacilli when examined microscopically were at first not touching, but evidently lying in a meshwork of agglutinated motionless flagella. By saturating the serum made from the motile strain with a suspension of the non-motile bacteria the somatic agglutinins were removed but the flagellar were left untouched.

Eisenberg and Volk (1902) found that at 60 to 62° C. the agglutinability of *B. typhosus* was impaired. In the same year Joos (1903) described two kinds of agglutinogen in *B. typhosus* and two corresponding agglutinins. The different manner of clumping associated with the two agglutinogens agreed with the observations on somatic and flagellar substances of Smith and Reagh. Joos also claimed that the antigen forming loose, floccular clumps was destroyed at 60 to 62° C., whereas the corresponding agglutinin was resistant at that temperature, and that the antigen which made small clumps proved resistant at 60 to 62° C., but its agglutinin was destroyed. Beyer and Reagh (1904), continuing the work on hog-cholera bacilli, found that the somatic antigen and flagellar agglutinin resisted a temperature of 70° C. for 20 minutes, but that a similar exposure damaged, though it did not quite destroy, the flagellar agglutinogen and the somatic agglutinin. They thus confirmed in a general sense the observations of Joos, but found that a higher temperature or a longer exposure were necessary to produce much effect on the heat-labile factors.

These observations did not carry the conviction which they deserved, partly because it was thought that the motile and non-motile strains of hog-cholera bacilli might belong to distinct 'species', although the somatic antigen was shown to be identical in the two strains. Also the non-motile strain was only agglutinated in a low dilution of serum and not with the flocculent bulky precipitate usually associated with specific agglutination in that class of bacteria. The experiments of Joos could not be verified by most observers probably because he used a temperature for testing lability which was found to be too low when his experiments were repeated by others.

Smith and TenBroeck (1915) showed that the agglutination reactions of the bodies of the bacilli of fowl-typhoid, *B. sanguinarium*, and of *B. typhosus* were identical and found the characteristics of the somatic clumping the same as in the case of the hog-cholera bacillus. They

emphasized the distinct difference between somatic and flagellar agglutinogens and agglutinins which they had previously described.

In 1916 Weil and Felix published their first paper on the *B. proteus* X 19 and its agglutinability by the serum of typhus patients. Later (1917, 1920), they differentiated the H form of this bacterium which grew with a spreading film or '*Hauch*' on agar and the O form (*ohne Hauch*), which formed isolated colonies and was especially related to the Weil-Felix reaction in typhus fever. The H form made large loose and also small granular clumps when acted on by agglutinating serum made from the rabbit with the H form. The O form made only small dense clumps with any serum which agglutinated it. They called the agglutinable substance present in the O form the O receptor, and the large flaking substance characteristic of the H form, the H receptor, and they pointed out that the H form had both receptors H and O, but the O form had only one. They published a large number of observations which led to the general acceptance of the H and O forms, and the two receptors which occur in motile species of bacteria. Attention was strongly directed to the subject by the importance of the Weil-Felix reaction with *B. proteus* X 19 in typhus fever.

At first it was supposed that the X 19 *proteus* bacillus did not react with the serum of typhus-fever patients unless it was living. Sachs (1917) showed that whereas the agglutinability of a suspension of X 19 motile bacilli was almost abolished by heating at 55° C. for 1 hour, heating at 80° C. or at 100° C. for 1 or 2 hours restored its agglutinability and clumping was then sharper and quicker than with unheated suspensions. The clumps were small and granular. This observation corresponds to similar ones by Porges (1905), Porges and Prantschoff (1906) and Eisler and Porges (1906), who found that heating at 100° C. restored agglutinability to a suspension of *B. typhosus* which had been made inagglutinable by exposure to 80° C. An inhibiting substance could be removed by washing from the emulsion heated at 80° C. In both cases the inhibition of agglutination is apparently due to a change in the H flagellar substance due to the heating, since it does not occur if an O (non-motile) culture is heated in the same way.

Bien and Sonntag (1917) found that the H antigen was destroyed by 30 per cent. or stronger alcohol at 37° C., but that the O antigen resisted this treatment and remained in very good agglutinable condition. Braun and Schaeffer (1919) showed that the H antigen was only present in motile cultures, but the O in both motile and non-motile. Growth on 0.1 per cent. phenol agar, or agar of poor nutrient quality, reduced or abolished the flagellar, leaving only O, antigen.

Weil and Felix (1917) found that the serum of rabbits inoculated with the H form of *B. proteus* X 19 contained agglutinins for both H and O antigens, and serum made from the O form had only a single small-flaking agglutinin which, like that in the serum of typhus patients, never clumped the H substance. Felix and Mitzenmacher (1918) often obtained pure

O sera with H emulsions heated at 100° C. They showed, too, that whereas the H agglutinins resisted a temperature of 70° C. though reduced by 50 per cent., the O agglutinin was completely destroyed at this temperature in half an hour, and that the O agglutinin in rabbit's serum corresponded to the agglutinins in the serum of typhus patients. The parallelism between the H and O forms of *B. proteus* X of Weil and Felix and the motile and non-motile forms of hog-cholera bacillus described by Smith and Reagh, and Beyer and Reagh, was, therefore, complete, and the H and O receptors corresponded exactly with the flagellar and somatic antigens of the American workers. The character of the clumps and the relative lability and stability of the antigens and agglutinins were also very similar, but the temperature required to differentiate the two antigens and agglutinins was not quite the same in the two classes of microbes. Orcutt (1924) returned to the examination of the hog-cholera bacillus and obtained the two forms, motile and non-motile, from a single strain. With these she demonstrated the separate and independent existence of somatic and flagellar antigens and antibodies and their different resistance to heat. She also obtained a flagellar emulsion which was agglutinable and when used as agglutinogen produced in rabbits flagellar, but no somatic, agglutinins. Jones (1927) showed that the critical temperature for inactivation of somatic agglutinins was 75° C. in the case of hog-cholera bacillus and of *B. abortus*. Weil and Felix (1918, 1920) and Weil, Felix and Mitzenmacher (1918) extended their work on the double nature of receptors and agglutinins to the *B. typhosus* and *paratyphosus* group, and showed that both H and O agglutinins are present in the serum both of patients and of rabbits immunized with the complete form of bacillus. It was found by Weil, Felix and Mitzenmacher (1918) that in this group of bacteria suspensions heated at 100° C. only evoked small-flaking agglutinins for the O form in rabbits, and White (1926) confirmed this in the case of *Salmonella*.

Subsequent work has shown that sometimes a certain amount of H or flagellar agglutinin is produced by the inoculation of the H form even when the suspension has been heated at 100° C. for 2 hours. This shows that, at any rate in such instances, the H or flagellar antigen has not been entirely destroyed although it cannot be recognized as an agglutinable substance.

Gruschka (1923) demonstrated the H and O forms of *B. enteritidis* and the two antigens named after these forms, and showed the very close similarity of the O antigen of *B. typhosus* to that of *B. enteritidis*. Goyle (1926<sup>1</sup>) further studied the relationship between the O antigen of the two species, and showed that it was probably not a single substance and was not completely identical in the two cases. Gruschka (1920, 1923) also confirmed the observations of Smith and TenBroeck (1915) on the identity of the O antigens in both human and fowl typhoid bacilli (*B. typhosus*, *B. gallinarum* and *B. sanguinarium*) ; so these organisms and *B. enteritidis* all have an O antigen in common. Felix and Robertson (1928) showed

that the motile anaerobes *B. tetani*, *B. chauvæi* and *V. septique* also have H and O forms and corresponding antigens and agglutinins with properties very like those in the previously examined motile groups, including *V. cholerae* investigated by Balteanu (1926). Arkwright (1927<sup>1</sup>) and Schütze (1928) have shown the same for *B. pseudotuberculosis rodentium*.

It has been pointed out by Felix (1924) that the systematic examination of each antigen separately which he has called the 'qualitative analysis of receptors' is the only satisfactory way of investigating the agglutination reactions of many kinds of bacteria. His work has been chiefly concerned with the differences between the H and O receptors or antigens. This part of the analysis can be carried out by testing the bacteria and their agglutinins before and after treatment of the antigens by heat or alcohol and the agglutinins by heating, and by the comparison of non-motile variants with the motile form.

The H (flagellar) and O (somatic) agglutinins, on account of their relation to the labile and stable antigens respectively, have been called 'labilotropic' and 'stabilotropic' by Schiff (1922), who reviewed the whole subject of agglutination (Schiff, 1925). The effect of heat on these two kinds of antibodies when contained in the same or in separate sera has been examined by Felix and Olitzki (1926) in a paper containing a summary of this subject and of the suggested relationship between these antibodies and the different serum proteins.

*Smooth or S and rough or R antigen.* It has been known for many years that spontaneously agglutinable cultures, especially of *B. typhosus*, do not always react with specific serum like the ordinary normal form, but this observation was difficult to make and the results were apt to be confused when 0·85 per cent. salt solution was used as the medium in the reaction, since the agglutination of the emulsions in this concentration of salt made the control of the stability of emulsions unsatisfactory (Savage, 1901; Nicolle, 1898; Walker, 1922; v. Lingelsheim, 1913). Arkwright (1914) showed that the spontaneous agglutination could often be avoided by the use of a weaker salt solution, and in 1921 described the 'rough' or R variants of *B. typhosus*, *B. dysenteriae*, &c., which formed irregular colonies on agar, grew in granules in broth and agglutinated spontaneously in salt solution, and in addition did not possess the same agglutinable substance and agglutininogen as the normal smooth culture. Much work has been done on the R form in the *B. typhosus-paratyphosus-coli* group by Schütze (1922), Goyle (1926<sup>1</sup>), White (1925, 1926, &c.), and others. In order to obtain stable suspensions a weak solution of sodium chloride (0·4 to 0·1 per cent.) must be used, or the bacteria extracted with alcohol (White, 1926).

The difference in the serological character of the R variant only affects the somatic antigen and the flagellar antigen is the same as in the S or 'normal' form. The flagellar (H) substance is present in the R form when this is motile. The R somatic substance does not react either in

agglutination, absorption or as agglutinogen in the same way as the S somatic antigen, but evokes in the rabbit R somatic agglutinins which agglutinate the R form and not the S, and can be absorbed by the R form only. This distinction between the S and R exists in the same way for other serological reactions (complement fixation, bactericidal action). The R somatic agglutinogen without flagellar substance is best examined as it exists in the non-motile R variant. The flagellar element can also sometimes be eliminated from a motile R suspension by heat or alcohol, but heating an R emulsion at 100° C. may have the effect of making it unstable even in distilled water (White, 1928); and the R antigen, after being heated at 100° C., is sometimes no longer susceptible to agglutination by the R agglutinin. When the motile R form is agglutinated by a serum containing antiflagellar agglutinins it always clumps rapidly in large, loose, characteristic flocculi like the motile S form, but when the R non-motile form is agglutinated, it usually forms much smaller clumps than the non-motile S form, and the deposit consequently is 'muddy' instead of 'sandy' and is dispersed readily by shaking, producing a uniform turbidity, although the minute clumps can still be demonstrated microscopically.

By some authors (White, Vol. IV, p. 86; Topley and Wilson, 1929) the term O is reserved for the somatic antigen when it is 'smooth' and for the smooth unflagellated form of the bacillus. The symbol  $\emptyset$  is used for the R form and R antigen, the degraded antigen of many American authors, which is deprived of its carbohydrate or specific soluble substance.

Schütze (1922) and White (1925, 1926) showed that the R antigen and agglutinin were not strictly specific, but appeared to be the same for a number of allied bacteria, e.g. R *B. typhosus* serum may agglutinate the R forms of *Salmonella*, some *B. coli* and some dysentery strains.

The three kinds of bacterial antigen which have been described in the Gram-negative intestinal group may all be present in a suspension of a pure culture of a single strain and presumably in a single bacterium and may be found in varying proportions. The flagellar antigen may be present in large or small amount, and the somatic antigen may be S or R or partly one and partly the other, since in a culture the change from S to R is often gradual.

The flagellar and somatic antigens were described as ecto- and endoplasmatic receptors respectively by Braun and Nodake (1924) and Hofmeier (1927). This designation is not a very happy one since there is no evidence that the flagellar material completely surrounds and covers the bacterium. It seems highly probable that the somatic antigen is on the surface, and that agglutination takes place as the result of sensitized points of somatic antigen coming together and cohering. It is possible that other kinds of agglutinogen may be discovered, e.g. the special antigen associated with endospores described by Mellon and Anderson (1919).

## PRECIPITATION.

The precipitation of bacterial extracts by specific precipitins contained in the serum of immune animals is probably allied to agglutination, as maintained by Paltauf (1897), Kraus (1897<sup>1 & 2</sup>) and Kraus and v. Pirquet (1902). Bacterial extracts free from whole bacteria may contain three or more kinds of precipitable substances.

*Flagellar Antigen.*

Flagellar antigen in a suspension of flagella has been described above. Such a suspension may give a precipitate with an antiflagellar (H) serum which has been diluted 1 in 3,200, and this will enmesh and carry down unrelated particles with it. Such an extract may completely lose its precipitability by filtration. It is the chief source of the observations on visible coagula of interbacillary substance in agglutinated emulsions.

*Somatic Antigen and Endotoxin.*

The kind of precipitable substance which is usually meant in speaking of the bacterial precipitin reaction is chiefly found in extracts obtained by filtering old broth cultures. This was especially dealt with by Paltauf and Kraus who used the reaction as a means of estimating the strength of *B. dysenteriae* 'toxin'. The precipitable substance consists probably of minute particles of the bodies of bacteria and contains somatic agglutinogen. It is generally heat-stable (100° C.) like the somatic antigen, and is probably the basis of the Ascoli reaction in anthrax. The precipitate obtained with such an extract and specific serum does not include and carry down to any considerable extent foreign bacteria or other particles if they are present. The chief difference between this kind of precipitation and somatic agglutination of larger bacterial fragments and whole bacteria lies in the fact that for the precipitin reaction it is necessary to use the antiserum very little diluted, e.g. 1 in 20 to 1 in 50, instead of a high dilution being sufficient as in agglutination. The explanation of this difference is probably to be found in the very small amount of precipitable substance present, and the need for the globulin in the serum—in order to complete a visible precipitate. The effect of added substances in making precipitation reactions more obvious has been pointed out by Bordet and Gay (1906), Dean (1912), Arkwright (1914<sup>1</sup>). The very close connection between the precipitin reaction, complement fixation and somatic agglutination has been emphasized by Weil and Felix (1920), Weil (1921), Felix and Robertson (1928).

*Chemically Prepared Fractions of Antigens.*

A substance precipitable by specific serum was obtained from *B. typhosus* by Porges (1905) in a manner similar to that used by him to render capsulated bacteria agglutinable. A thick emulsion was first treated with an equal quantity of hydrochloric acid, N/1 to N/4, and then heated at 100° C., cooled and neutralized with sodium hydrate. The

substance precipitable by specific serum which was obtained, no doubt contained part of the somatic antigen of the bacillus, including a material corresponding to the 'specific soluble substance' of Heidelberger and Avery.

More exact chemical analysis of bacteria has enabled two classes of substances, protein and carbohydrate respectively, to be isolated. Both appear to be associated with the somatic antigen, and no component referable to the flagellar or heat-labile antigen has been isolated. The protein substance is truly antigenic and evokes antibodies, it is also precipitated by these and by certain antibacterial sera, but it does not as a rule appear to be strictly peculiar to the type or species from which it has been isolated.

The carbohydrate substance 'C' yields a high percentage of reducing sugar and gives highly specific type or species reactions with specific antisera versus the whole bacterium. It is apparently responsible for the differences between nearly related cultures of different types. In its pure state, i.e. unless combined with protein, it is unable to stimulate the production of antibodies. Such non-protein non-antigenic substances which precipitate with specific sera have been called by Landsteiner 'haptens'. In the case of the pneumococcus the substance 'C' is found only in the capsulated virulent form (Heidelberger and Avery, 1923, 1924; Reimann, 1926), and not in the 'degraded', non-capsulated and non-virulent or R form which contains the protein substance alone. Similar results have been obtained with the pneumobacillus of Friedländer (*Encapsulatus pneumoniae*) by Heidelberger, Goebel and Avery (1925) and Julianelle (1926). The substance 'C' is chemically different in the different 'types' of the pneumococcus and of the pneumobacillus.

An important difference between the substance C in Types I, II and III of pneumococcus consists in the sugar which can be derived from each. That an apparently slight difference in the structure of the carbohydrate molecule can affect the specific serum reactions has been further demonstrated by Goebel and Avery (1929) and Avery and Goebel (1929). These workers synthesized protein-carbohydrate compounds from glucose and galactose with both egg-albumin and serum-globulin, and found that the four compounds obtained produced different antibodies in rabbits, according to the carbohydrate entering into the composition of the antigen. The two sera made with glucose compounds precipitated both glucose-protein substances but not the galactose-protein substances, and vice versa. These reactions were apparently independent of the particular protein from which the antigen was made—whether egg-albumin or globulin—whose special character did not influence the reaction.

Heidelberger and Kendall (1929) take the view that the precipitin reaction with the soluble specific substance of pneumococcus Type III can best be explained as a chemical reaction between the soluble bacterial products and the precipitin in the antiserum, and that the 'zone phenomenon' which occurs is due to the solubility of the precipitate in excess of the specific soluble substance. They base this conclusion, which

has also been put forward by previous workers, on careful quantitative measurements of highly purified preparations of the two reacting bodies and the precipitates.

In the *typhosus-coli* and in the *Staphylococcus* groups carbohydrate specific precipitable substances have been recognized by Zinsser and Parker (1923), White (1926) and others. Laidlaw and Dudley (1925) obtained a carbohydrate substance precipitable by specific serum from *B. tuberculosis*. Landsteiner and Levine (1926, 1927) and Furth and Landsteiner (1928) have analysed and compared the antigenic fractions of *B. typhosus*, *B. paratyphosus B*, *B. enteritidis* Gaertner, *B. proteus* and *V. cholerae*. Their experiments resulted in the discovery of two protein substances, P1 and P2 in *B. typhosus*, both soluble in 75 per cent. alcohol and a substance, C, containing a high percentage of carbohydrate and a trace of protein. The proteins were very active in the production of antibodies. P1 was precipitated by antisera made with *B. typhosus*, *B. paratyphosus B* and *B. coli* and Gaertner's bacillus but not by serum from *B. proteus* or *V. cholerae*. P2 reacted with *B. typhosus* and *B. paratyphosus* only. Neither protein appears to correspond to the flagellar heat-labile antigen. The substance C is much more highly specific than P1. Cross-precipitation, however, occurs of the C substances derived from *B. typhosus* and *B. enteritidis* Gaertner by the antisera to both these bacteria, and the C substance from *B. paratyphosus B* is only slightly affected by serum from *B. typhosus*. The results with the precipitation of C agree with the previously known facts about cross-agglutination of somatic antigen in this group. These workers found no lipoid substances in their extracts. White obtained a protein reaction and no evidence of carbohydrate in emulsions of R salmonella, as opposed to the result with S emulsions of the same strains, with which he got no protein reaction, but an indication of the presence of carbohydrate under the same conditions. He also found that the R antigen as shown by agglutination was more widely distributed and much less specific than the S antigen.

In the *Streptococcus* family the number and characters of the antigens have not been completely defined, but Lancefield (1928) and Lancefield and Todd (1928) describe three fractions of the antigen. One, which is protein and is contained in all the members of the group including *Streptococcus viridans*, *pneumococcus* and *S. mucosus*; the second is non-protein and serves to differentiate the haemolytic streptococci from pneumococcus and the rest; the third, 'M', is more highly specific and distinguishes different types of *S. haemolyticus*, and is also protein in composition. Substance 'M' is present in all the 'matt' or dull-surfaced colonies of haemolytic streptococci whether they are virulent or not, but it is absent from the 'degraded' and 'glossy' colonies which are always non-virulent.

Different kinds of antigen have not so far been clearly distinguished in other non-motile bacteria, but Nicolle, Jouan and Debains (1919) differentiated labile and stable precipitable substances in *Gonococcus* and *Meningococcus* by using the method of Porges.

*Exotoxin.*

Another kind of precipitation of bacterial filtrates by specific antiserum is that of certain true exotoxins (and perhaps some endotoxin), by antitoxin as in Ramon's reaction of diphtheria antitoxin with toxin and anatoxin or toxoid.

Schiff (1925) has summarized the literature on the relation of agglutination to precipitation.

**MULTIPLE FLAGELLAR AND SOMATIC ANTIGENS.**

It has been seen that the somatic S antigen may be divided chemically into distinct fractions. Each of these fractions may be recognizable by its special serological properties and may form a precipitate with antisera, and in some cases stimulates the production of antibodies, when inoculated as antigen.

In addition to the complex character of some antigens due to their chemical composition, it has been found that the same bacterium may contain two or more flagellar or multiple S somatic antigens. Each of these antigens (flagellar or S somatic) may consist of a mixture or 'mosaic' of different type antigens. These are revealed by their reactions with different specific sera and serve to separate allied races into different 'types'. Different strains contain the antigens in different proportions and these are represented in the corresponding agglutinins, though the relative proportions of antibodies may not be exactly the same as of the antigens. Different types are detected by agglutination, and these compound antigens and the races or strains containing them have also been elucidated by the absorption technique.

***Qualitative Serological Analysis of the Antigenic Complex.***

Whether the flagellar or somatic antigens in a given strain and the agglutinins in the corresponding serum are single or multiple may be tested in various ways : (i) by making an agglutinating serum and finding out how many different strains of bacteria can be agglutinated by it, further, (ii), by making sera versus all these different strains and determining how far they are able to agglutinate the same series of suspensions, and (iii) by absorbing from these sera the agglutinins for the different strains.

***Agglutination.***

If some of the strains which are agglutinated by a single serum lack a considerable part of the antigen which has been used to produce it, and others lack another part, they will probably not all be equally agglutinated by each other's sera.

In order to observe all the factors in agglutination reactions it is advisable to eliminate the flagellar antigen by heat or alcohol before testing the somatic agglutination, or the more bulky flocculation may interfere with the observation of the small granular clumps.

Strains are often met with containing an antigen which is also present in a large number of allied cultures and produces agglutinins for them all,

and if this common antigen is held in relatively large amount it may mask minor differences and even cross-agglutination may not reveal well-marked antigenic differences which really exist. It is in such cases as this that absorption of agglutinins proves of value. It is, however, commonly found even in the case of very closely related strains, e.g. among strains of *B. typhosus*, that agglutinating serum for one does not have an equal effect on all the strains, and is usually more effective on the strain used to produce it.

The results of examining a group of less nearly allied races such as exists in the salmonella family may be very similar. In these cases sera made from some strains do not give good reactions with an emulsion of the type strain, or they may almost fail to agglutinate it at all, though all the strains are well agglutinated by the serum of the type. Such an occurrence is often rather quantitative than qualitative and due to the type strain being better endowed with flagellar antigen, or to the flagella being completely absent from some other strain, or again, the defect may reside in the somatic antigen. On the other hand, there may be differences in kind between some of the individual component antigens in the different strains.

#### *Reciprocal cross-agglutination.*

The different behaviour of allied strains and their sera when tested reciprocally was made the basis of the doctrine of principal (Haupt) and group (Gruppe and Neben) agglutinins. The group agglutinins which acted on more or less related strains were believed to be of a different nature from the principal antibodies and were supposed to be produced by a kind of overflow from the production of the latter. It is more probable that they often only differ from the agglutinins which react solely with the homologous strain by being present in smaller quantities, as the result of the producing strain containing smaller amounts of the corresponding antigens. Principal and group agglutinins may be either flagellar or somatic, and heterologous strains may resemble or differ from the strain which has produced the serum either as regards flagellar or somatic antigen and either qualitatively or quantitatively.

It seems, therefore, that the whole of these phenomena can best be explained by the existence of a number of antigens present in the different strains in varying quantities and relative proportions, and all represented by agglutinins in quantities roughly proportionate to the amounts of antigen present in the suspension used to stimulate antibody production. This multiplicity constitutes the so-called antigen mosaic in the individual bacteria.

#### *Absorption of agglutinins.*

The technique of absorbing agglutinins was used experimentally by Bordet (1899), Joos (1901) and Eisenberg and Volk (1902). It was introduced for clinical purposes by Castellani (1902) in order to distinguish a multiple infection from one due to a single micro-organism which had

produced non-specific or group, as well as specific, agglutinins in the blood. In the case of a single infection the actual infecting agent absorbed all the agglutinins present, whereas other organisms could only bind the group agglutinins.

The method has been developed and used extensively for antigenic analysis by many workers in different groups of bacteria. Boycott (1906) and Bainbridge (1909) applied the technique to the classification of the *B. paratyphosus* group, and Schütze (1922) and White (1925, 1926) made long series of investigations in the *Salmonella* group. Andrewes and Inman (1919) made a very thorough analysis of the antigenic constituents of different races of mannitol-fermenting dysentery bacilli. Similar technique has been employed for classifying meningococci and gonococci, especially by Gordon and others (1920), Gordon and Murray (1915), Torrey (1907) and Griffith (1916), and by Schütze (1928) for investigating *B. pseudotuberculosis rodentium* and by Cornelius (1929) in the case of *Pasteurella* strains.

For absorption of agglutinins a rather low dilution of serum is used, say 1 in 100, of a serum with a homologous titre of 1 in 5,000. To this dilution is then added an equal quantity of a thick suspension of bacteria. After incubating the mixture for half an hour and allowing it to stand in the cold till agglutination is completed it is centrifuged clear and the supernatant is again tested for agglutinins. If the emulsion contains R bacteria, it is best to carry out the absorption in the minimum concentration of salt which will ensure specific agglutination, e.g. 0·1 or 0·2 per cent. NaCl. It is found almost always that the homologous strain, if used in sufficient quantity, removes all the agglutinins, but other strains which are also agglutinated often remove only a part of the agglutinin complex, leaving behind other agglutinins which are capable of clumping the homologous strain almost or quite to the original titre. Other strains, again, which have antigens in common with the homologous but not with the absorbing strain are also clumped by the remaining agglutinins.

By this means very complex and to some extent quantitative relationships between different races of allied bacteria have been established (Andrewes and Inman, 1919; Schütze, 1928; White, 1925). The technique of absorption of agglutinins requires careful execution and interpretation, otherwise mere quantitative differences may be made to appear as though qualitative. After being absorbed, serum contains unagglutinated and unprecipitated particles and substances in solution from the absorbing bacteria which may have some influence on subsequent reactions. Felix stresses the importance of using for absorption suspensions of bacteria which contain as little free bacterial substance as possible, and considers that the bacteria should be washed before use for this purpose (Weil and Felix, 1920). Washing may, however, have the effect of removing some of the flagella. Weil (1905, 1911) recorded experiments showing that strong bacterial extracts sometimes had a marked inhibiting effect on

agglutination and that this was not always confined to extracts of bacteria for which the agglutinins were specific. He believed that the extracts converted the agglutinins into 'agglutinoids', but the existence of the latter bodies is much controverted and has not been conclusively demonstrated. In spite of these drawbacks consistent and sharply defined results can frequently be obtained by the absorption technique in expert hands. It is important that the extent and significance of the simple and reciprocal agglutinations should be thoroughly explored before resorting to absorption. It has been pointed out by Schütze and White that no safe conclusions as to antigenic relationships can be drawn by this method without making antiserum for each strain under investigation and performing cross-agglutination and cross-absorption with both strains and sera. This has been called the 'mirror' or reciprocal absorption technique.

The chief object of the method is to decide whether two strains are completely identical antigenically or merely contain sufficient antigen in common to give approximately the same reactions with reciprocal agglutination tests. It may be used in the analysis of both flagellar or somatic and S or R differences.

The results of absorption are, therefore, of value in differentiating apparently similar strains and subdividing groups which have been made by means of ordinary agglutination tests, or on the other hand, to confirm the identity of strains which are probably the same, but may have certain perhaps trivial differences.

#### *Quantitative Agglutination.*

In using agglutination for the diagnosis of disease it was very soon found necessary to make exact observations of the highest dilution of the patient's serum which would clump the bacteria, because in some normal sera agglutinins are present which produce clumping in low dilutions. It is, therefore, necessary to ascertain the minimum concentration which can be regarded as giving a significant result in diagnosis. In determining this criterion the agglutinability of the particular culture of the bacillus which is used has to be taken into account. Since normal agglutinins for *B. typhosus* and *B. dysenteriae* are not usually effective in a dilution higher than 1 in 100, this fraction or a weaker dilution has been usually regarded as significant. The criterion, however, varies with the kind of disease investigated, and the character of the suspension of bacteria. When agglutination is used to determine the nature of an unknown bacillus by means of a specific agglutinating serum similar precautions must be observed. The titre of the serum and the range of its activities on allied strains of bacteria must be previously ascertained and the agglutinability of the culture by normal and if possible by several immune sera should be examined. For whichever purpose agglutination has been used it has been customary to determine the highest dilution (weakest serum) which will clump the suspension. When the serum is not much diluted a range or

zone of dilution sometimes is found in which clumping does not take place, which is a reason for using at least several dilutions at fairly wide intervals.

When an unknown bacillus is examined with a specific agglutinating serum, it may be important to use the serum near the upper limit of its titre, since agglutinins for allied races (group agglutinins) are often present in addition in the lower dilutions. These possibilities of error are to a great extent avoided by obtaining a thorough preliminary knowledge of the agglutinin content of the test serum.

Formerly the whole stress in agglutination was laid on the quantitative features of the reaction and the qualitative side was ignored unless absorption was performed. As a rule it was only the reaction of the predominant antigen and agglutinin present which was observed and measured, by estimating by dilution the titre of the agglutinating serum.

Since no difference between somatic and flagellar agglutination was recognized even if both were present, the results were due to the combined effect of the two factors and were often very confusing in consequence. If flagella were present usually only the flagellar agglutination was noted. The occasional disconcerting absence or poverty of reactions due to variations in the development of flagella and motility of the culture appeared to be quite unaccountable. When only one serum was used to test a culture and the presence of flagellar and somatic antigens of different qualities in a single culture was not recognized, the titre or dilution of the serum in which agglutination occurred was the only serological means of distinguishing cultures. Accordingly very great importance was attached to the quantitative factor and the titre of agglutination.

By means of this criterion the rate and manner of production of antibodies in the animal body have been very carefully studied and the best method of obtaining highly agglutinating sera has been investigated. Some of these studies are of great value, but it is unfortunate that a great deal of the most careful work was done with motile bacteria, *B. typhosus*, *B. coli* and *V. cholerae*, and in some instances the unrecognized presence in variable proportions of two or three kinds of antigen has interfered with the clearness of the results.

Quantitative estimation of the titre to which a serum reacts with a known antigen, or known serum with an antigen or bacterium under examination, is essential in many investigations. It is important that the test serum or antigen should be standardized as far as possible as regards its components and their sensitiveness to reaction under standard conditions. The importance of the standardization of agglutinable suspensions has been emphasized by Dreyer (1909) (see also Dreyer, Walker and Gibson, 1915, and Gardner, 1918) and the need for the preparation, by means of absorption, of sera with simplified agglutinin content and containing as far as possible single agglutinins has been urged by Andrewes (1925).

The titre to which a known serum will agglutinate a bacterial suspension is to some extent a measure of the antigen content of the bacteria, and the

observed titre of a serum shows within limits the degree of community between the antigen of the strain used in preparation of the serum and the emulsion under examination.

In absorption experiments the comparison of the titre before and after absorption of a serum is particularly necessary. It is desirable in such experiments to use a serum of high titre so that the more and the less important antigens may all be represented in proportion. It has, however, been found that the agglutinins in a serum resulting from numerous inoculations of a rabbit are less likely to show a correct reflection of the bacteria inoculated, since an antigen present in lesser proportion may have provoked an undue amount of antibody. When the bacteria concerned are known to have certain distinctive characters, quantitative tests are sometimes unnecessary and qualitative search for special antigens or agglutinins may give equally valuable results.

#### *The Practical Value of Qualitative Analysis.*

Since the multiplicity of antigens in a single bacterium has been recognized it is realized that the presence or absence of one or two antigens will often give a decisive diagnosis of a culture or that the corresponding agglutinins in a serum will decide the 'species' of the infecting organism, and exhaustive quantitative examinations, though very valuable in experimental work, are frequently unnecessary for diagnostic purposes.

#### *Determination of bacterial cultures.*

*Single and multiple antigens ; antigenic mosaic.* A bacterium may possess only one recognized agglutinogen. A smooth culture of *B. dysenteriae* (Shiga) may only contain one agglutinogen and only one agglutinin is to be found in the corresponding serum. If such a culture takes on the R characters, the R is substituted for the S somatic antigen and agglutination does not occur with the S serum more than with normal serum, but the R serum contains the special R agglutinin. A partially R culture contains both S and R somatic antigens. The S agglutinable substance resists a temperature of 100° C. for one hour, and an emulsion so heated is suitable as inoculum for making an S agglutinating serum. The R somatic agglutinable substance is less heat-resistant. The agglutination of the mannitol-fermenting strains of *B. dysenteriae* is more complicated, since, whilst the antigen and agglutinins are all somatic, either S or R, the S antigen is often complex and consists of several units forming what has been called the antigenic mosaic. Five or more different races of this 'species' have been described by Lentz (1912) and Andrewes and Inman (1919). One well-known Y strain of *B. dysenteriae* contained three or four S antigens, which included all or most of those found in different combinations in the various strains described, such as the V, W, X, Y and Z strains of Andrewes and Inman. These workers analysed the antigenic content of a number of different strains by agglutination with separate sera prepared from them, by testing the

antigenic properties of the individual cultures in rabbits and by absorbing the agglutinins with different strains and estimating the agglutinins left both qualitatively and quantitatively. It was possible to absorb the agglutinins independently and so to obtain sera containing only one or two remaining agglutinins. Gordon and Murray (1915) and Griffith (1916) studied the meningococcus by similar methods and differentiated several types, and the gonococcus has been examined by the same means with similar results (Torrey, 1907; Arkwright, 1911). Similar work has also been done but not with uniform success in other groups of bacteria.

The phrase 'antigenic mosaic' has been used in a more or less metaphorical sense for the complex composition of the antigen in certain bacteria. It is possible that, at any rate in the case of somatic antigen, the idea of a surface pattern may not be far from the truth. Since agglutination is due to the cohesion of similar surfaces on bacteria and since these surfaces may be of different kinds as shown by their sensitization by different agglutinins, a variety of antigenic substances must be located each at separate spots on which the various agglutinins are deposited and between which the cohesion occurs. That the independent 'sensitization' of different points on the surface of a bacterium may occur, the following experiments by F. S. Jones suggest.

It had been shown that collodion particles or bacteria could be given a special sensitive surface by treating them with various protein substances, after which they could be agglutinated under conditions which would precipitate the protein (Arkwright, 1914<sup>1</sup>; Loeb, 1922-3). Jones (1927) found that particles had become specifically sensitized by a protein with which they had been in contact. He showed this by effecting their agglutination by means of a serum made versus the particular protein concerned. He also showed that particles could be sensitized at the same time to five different protein substances, egg-albumin, cow-serum, horse-serum, rabbit-serum and chicken-serum, and could, after washing, then be agglutinated by any one of the antisera for these proteins. If the protein had been sufficiently removed from the suspending medium this experiment appears to demonstrate that none of the proteins completely coated the particles, but that they must have been present on their surfaces as 'adherent patches'. The presumed adsorption of serum protein which follows the action on bacteria of specific antibodies is more regular, perhaps affects larger surfaces, and the union is certainly much firmer.

When agglutination tests with different absorbed and unabsorbed sera are performed with flagellated bacteria the results are more complicated and their interpretation proportionately difficult. In the *Salmonella* it has been shown by Schütze (1922), White (1925, 1926) and others that both the flagellar and somatic antigens may be multiple. The analysis and determination of antigenic composition of different allied races shows that in some cases the somatic (as in some *Salmonella* strains) and in other the flagellar antigen (*B. proteus*, *B. pseudotuberculosis*)

*rodentium*) forms the connecting link between different races or species. Conversely, the more specific of these antigens forms the criterion for discriminating between races or species.

*Specific and group phases.* Besides the differences in antigens found in different races Andrewes (1925) showed that the same culture of a pure strain of *Salmonella* often contained two sets of individual bacilli with very different antigens, the one specific for the particular race, the other with wide affinities for a whole group of allied races of *Salmonella*. He also showed that these two phases, which he called 'specific' and 'group', could be separated, nearly pure, by picking different colonies of similar appearance off an agar plate, but that both phases often changed speedily, and after a day or two in broth culture each gave rise again to both phases. For the purpose of examining this phenomenon Andrewes advocated the use of specific and group sera purified by removing by absorption the agglutinins which were not required. White (1925) showed that these phases were concerned with changes in the flagellar antigen, the somatic antigen being the same in both.

#### *Qualitative analysis in the diagnosis of disease.*

The practical application of the analysis of antigens and agglutinins has been especially worked out by Weil and Felix. As is well known, these workers in 1916 isolated from a case of typhus fever a strain of *B. proteus* which was agglutinated by the serum of typhus patients but not by that of other persons. They subsequently isolated several similar strains which they called *B. proteus* X. Of these, X19 was found to be the most serviceable since it agglutinated with all typhus sera, often to a high titre. They also obtained from this strain a non-motile or O form and showed that it was the O or somatic antigen which was peculiar to the X strains, and that the H or flagellar antigen which was shared by a number of different strains of *B. proteus* was of no diagnostic value, and that agglutinins for it were not present in typhus blood.

Felix and Olitzki (1926), Felix (1924) and Olitzki (1928) have brought forward strong evidence that the diagnosis of typhoid fever may be made by attention to a qualitative agglutination by the patient's serum. If the patient is unvaccinated with typhoid or T.A.B. vaccine and has not previously suffered from one of the enteric diseases, flagellar agglutination of *B. typhosus*, *B. paratyphosus A* or *B* makes it certain that he is infected with the bacillus agglutinated, as was formerly concluded from a positive Widal. The diagnosis is certain even when the agglutination titre is quite low, e.g. 1 in 100 or 200, and there is no need to look for a rising titre, but this result usually is not obtained till the tenth or fourteenth day.

The serum of an enteric-fever patient not only agglutinates the flagellar (H) antigen, but also from about the end of the first week of fever till a few weeks after defervescence agglutinates the somatic antigen, i.e. produces granular agglutination of a heated (100° C.) or alcoholized suspension of the infecting organism in a dilution of 1 in 50 to 1 in 500.

Since the somatic agglutinin in an infection with either *B. typhosus*, *B. paratyphosus A* or *B* or *B. enteritidis* Gaertner will sometimes agglutinate any of these four bacteria, the presence of somatic agglutinins in patients' serum cannot distinguish between these infections for certain. It is, however, very strong evidence that an infection with one of them exists, since vaccination, even if recent, produces only flagellar, or merely a trace of somatic agglutins in very low titre. If the patient has been recently vaccinated the presence of flagellar agglutinins corresponding to the vaccine is of little or no importance, and therefore, somatic agglutination in these cases, though less specific, may be of very great diagnostic value. For this test a suspension with a sensitive and purely S somatic antigen is required and an alcoholized suspension of a smooth culture of *B. enteritidis* Gaertner is, perhaps, the best for the purpose, since Gaertner's bacillus is often better endowed with S somatic antigen than most strains of *B. typhosus*, and their somatic antigens are almost identical. If an alcoholized suspension of *B. typhosus* is used it is very important that a suitable strain with well-marked sensitive S somatic antigen should be selected. Felix's strain 901 has been found very satisfactory. The subject of agglutination in diagnosis of typhoid and paratyphoid fevers, and the unreliability of flagellar agglutination in persons vaccinated against these diseases has been recently dealt with by Felix (1929) and Gardner (1929). The value of qualitative receptor analysis in the investigation of the enterica group of diseases has been reinvestigated and confirmed by Stuart and Krikorian (1928). Pijper and Dau (1930) find that oral immunization of man with typhoid vaccine produces O (somatic) but no H agglutinins.

It has been the experience of several observers (Felix, 1924; Pijper, 1923; Pijper and Pullinger, 1928) that a certain percentage of cases of typhoid fever from which *B. typhosus* has been isolated do not give a good Widal reaction as tested in the ordinary way. No distinct agglutination occurs with the customary diagnostic emulsion in the short time usually allotted to this test (2 or 3 hours) or even in some cases during a longer period. Felix has found that these patients have no flagellar agglutinins in their blood, but that somatic agglutinins are present and can be demonstrated if an emulsion of a suitable strain with a sensitive somatic antigen is used for the test, and a longer time (18 hours) is allowed for the clumping to take place. If the test emulsion or serum contains formalin or phenol the somatic agglutination is usually inhibited in some degree.

#### BACTERIAL SUSPENSIONS FOR AGGLUTINATION.

Bacteria for agglutination may be used either as cultures in broth or suspended in salt solution after being grown on the surface of agar. Cultures on solid media have the advantage that the suspension contains less adventitious matter and the bacteria can more readily be obtained in large quantities; also a uniform emulsion is sometimes more easily

secured and the salt content can more readily be varied at will. Such suspensions are, therefore, commonly used for experimental work. Broth cultures have, however, certain advantages and have been used much more for diagnostic purposes (Dreyer, 1909; Ficker, 1903). The bacteria in liquid cultures are generally more motile and better supplied with flagella than those from solid media. This is important when it is desired to detect or estimate the flagellar, i.e. the H or heat-labile antigen.

In the original microscopic method of Gruber and Durham (1896) and in Widal's diagnostic technique, the cessation of motility was described as the first stage in agglutination of *B. typhosus*. Bordet (1896) also described cessation of movement as the result of adding a small quantity of specific serum, inactivated by heating at 60° C. for 30 minutes, to cultures of cholera vibrios. These observations were made with living broth cultures and are important since they indicate the share which flagella take in agglutination (Smith and Reagh, 1903; Arkwright, 1927<sup>2</sup>).

The temperature at which the culture is grown sometimes has a marked effect on the agglutinability of the emulsion. Nicolle and Trenel (1902) found a strain of *B. typhosus* which was non-motile and 'inagglutinable' when grown at 37° C., but motile and agglutinable in cultures kept at laboratory temperature. The same is true of *B. inconstans* (Braun and Löwenstein, 1923) and *B. pseudotuberculosis rodentium* (Arkwright, 1927<sup>1</sup>). The addition of 0·1 per cent. phenol to nutrient agar was found by Braun and Schaeffer (1919) to inhibit the production of flagella and the corresponding heat-labile antigen. A similar effect resulted when culture media with very scanty nutrient were used. A careful adjustment of the pH of the media has been found to affect the agglutinability of different kinds of bacteria, e.g. meningococcus (Elser and Huntoon, 1909). In the case of the last-mentioned microbe it has also been found important to exclude as far as possible traces of culture media, even water of condensation, from the suspension (Murray, 1929).

If flagellar agglutination is being tested, it is well to be sure that the culture used is highly motile. If smooth somatic antigen is required it must be ascertained that the suspension is not agglutinated by salt solution, and, whilst agglutinated by an S somatic serum, is not affected by a serum versus the R somatic antigen.

If it is desired to detect R antigen, spontaneous agglutination in 0·85 per cent. saline is in some cases a sufficient test for the suspension but agglutination with an R somatic and not with an S somatic serum is the surest criterion, and some degree of agglutination with normal rabbit-serum is often evidence of the presence of R antigen.

#### *Medium for Suspending the Bacteria.*

In order that agglutination may take place a small amount of neutral salt must be present. It was formerly customary to use a solution of sodium chloride of 0·85 per cent. for making the suspension and for diluting the serum. As a general rule it is better to use distilled water

for the suspension and to add the necessary salt with the serum. Since many bacteria agglutinate spontaneously in 0·85 per cent. or weaker sodium chloride solution, it is best as a routine to dilute the serum with 0·85 to 0·2 per cent. saline so that when mixed in equal parts with the distilled water suspension the concentration of sodium chloride is 0·4 to 0·1 per cent. A final concentration of 0·1 per cent. is quite enough to allow specific agglutination to occur even with very dilute serum, though the titre of the serum may be slightly lower with 0·1 than with 0·4 per cent. (Arkwright, 1914<sup>1</sup>, 1921; White, 1925, 1926). In order to obtain uniform and permanent suspensions of some bacteria, e.g. *C. diphtheriae*, Ringer's solution has sometimes been found preferable to sodium chloride.

#### *Living, Killed and Preserved Suspensions of Bacteria.*

Living cultures may be replaced for ordinary purposes by dead suspensions from agar. Some bacteria rapidly autolyse in salt solution or distilled water, and heating at 56° C. for 30 minutes is necessary to prevent this. For flagellar agglutination these may be killed by heat at 52 or 56° C., but even at these temperatures the agglutinability may be considerably reduced. The addition of formalin 0·1 per cent. as recommended by Dreyer (1909) and subsequent exposure to 37° C. for some days till dead is probably the best method, but 0·5 per cent. phenol added to an emulsion prepared from an agar growth is very satisfactory.

For somatic agglutination the suspension must be kept without formalin, carbolic, &c., since either of these preservatives has been shown by Felix and Olitzki (1928) to inhibit somatic agglutination in the presence of H or flagellar antigen. Thirty per cent. alcohol as recommended by Bien (1924) is found very useful; the suspension then contains no agglutinable flagellar antigen.

#### *Preservation of Serum.*

The agglutinating serum is best kept unheated and without the addition of phenol, since this often interferes with somatic agglutination. It may be kept either sterile and untreated, or mixed with an equal quantity of glycerin.

#### NORMAL AGGLUTININS.

The existence of agglutinins has been frequently observed in the blood of normal animals for some bacterium with which the animal is not known to have been infected. The dilution of serum showing this phenomenon is usually very low, e.g. 1 in 20 or 1 in 150. In many of the cases observed the clumping has, no doubt, been due to a partial R change in the bacteria used for the test emulsion. This may have caused agglutination by a non-specific reaction between the serum and the R strain (White, 1928), or the wide community of R antigens among different strains may have led to the production of R agglutinins in the animal body by an unsuspected infection; small amounts of specific smooth somatic agglutinins, however, may occur normally. Normal

agglutinins are nearly always somatic (O), and, as shown by Felix (1929), are heat-labile like the somatic antibodies in immune animals. In the ox and occasionally in the horse, normal flagellar agglutinins may be found (Schiff, 1925).

#### PARAGGLUTINATION.

Kuhn and Woithe (1909) described a phenomenon which they called 'paragglutination'. They sometimes observed the agglutination of *B. coli* and cocci with the serum of patients infected or recovering from infection with *B. dysenteriae* (Flexner). The property of paragglutination rapidly disappeared when the bacteria were subcultured. They believed that in some way the colon bacilli, &c., had become susceptible to the specific agglutinins for the dysentery bacilli by growing in the intestine of the patient. The explanation of this phenomenon is doubtful, and it has seldom been observed with sufficient distinctness to merit confident acceptance. The heterologous agglutination of R or partially R strains by R agglutinins of only moderate specificity needs to be excluded. This possibility demands attention when observations on similar occurrences are made in the future.

#### THE PRODUCTION OF AGGLUTININS AS THE RESULT OF INFECTION OR VACCINATION.

Agglutinins, like other antibodies to bacteria or other protein substances, are produced chiefly as the result of parenteral inoculation of the antigenic material. It is found that they are formed most regularly and to a higher titre if the antigen is injected intravenously. They may also appear in the circulation in more irregular and lesser amounts after ingestion of the antigen by the mouth. It is partly because the intravascular route is the most successful and partly because the site of formation of antibodies cannot for other reasons be attributed to any special locality in the body that it is generally believed that some widely distributed tissue is responsible for their manufacture. The cellular reaction which follows inoculation or infection is most constantly concerned with the reticulo-endothelial tissues and the bone-marrow, and especially with the former; consequently it is probable that the site of antibody formation is to be placed in the so-called 'reticulo-endothelial system'.

This view is supported by the local inflammatory reaction and increase in the endothelial cells in the subcutaneous tissues which follows the injection of antigen into this and similar sites. The production of antibodies by the tissues results in the development of a new function by which the tissues continue to form these substances often for many months or even years and in addition the important condition is established that further introduction of the same antigen leads to renewed formation of antibodies of the same kind, which enter the circulating blood sometimes in a higher concentration than after the first inoculation. This phenomenon is utilized in producing agglutinating serum in the

laboratory by giving repeated injections of the agglutinogen at suitable intervals. The restimulation of the antibody formation is very important in resistance to disease, since a former attack or inoculation leaves behind a source of supply which at an early stage of reinfection pours out a fresh wave of antibody and in this way averts the disease although antibodies may not be recognizable in the circulating blood before the fresh entry of antigen.

The production of agglutinins to the flagellar and somatic antigens is so far different that while the former is much more readily stimulated in the first instance, or at any rate is much more easily recognized in the blood, and is more readily caused to reappear or increase in amount by subsequent introduction of the same antigen or even by a non-specific stimulus, the somatic agglutinin seldom reaches so high a titre during infection and in some cases, e.g. *B. typhosus*, more rapidly disappears after recovery and is not known to be capable of resuscitation by non-specific stimuli.

The difference between the two kinds of agglutinins has been chiefly studied in enteric fever and salmonella infections and in the case of agglutinins to *B. proteus* found during typhus-fever infections. The subject has been critically reviewed by Felix (1929). The evidence in many diseases, including typhoid fever, is often complicated by the persistence of infection in a subacute or chronic form as in bacillary carriers in enteric, dysentery, Malta fever, bovine abortion, &c.

The occasional long persistence of agglutinins in the blood-serum of these diseases is well known.

The evidence for or against non-specific restimulation of somatic agglutinin formation after infections with non-flagellated bacteria has not yet been thoroughly investigated.

#### THE VALUE OF AGGLUTINATION AS AN AID TO CLINICAL DIAGNOSIS.

It has in the past been accepted as a general principle that when a patient has been infected for a week or more with a special bacterium a diagnostic agglutination reaction with his serum may be expected, and a similar effect has been found to hold in many different species of animals. Experience has, however, shown that there are many exceptions to this rule. Amongst vertebrates satisfactory specific agglutination has not been observed in cold-blooded animals, but the serum of birds may contain significant specific agglutinins. Certain mammals—monkeys, rabbits, horses, asses, mules, oxen, sheep, and rats—have all been shown to yield agglutinins after infection or experimental inoculation, but rats produce far less potent serum than rabbits, which are particularly active in this respect. Guinea-pig serum, on the other hand, as a rule contains agglutinins only in a very low titre.

The value of agglutination for the diagnosis of disease varies also very much with the kind of micro-organism which causes the infection. A patient's blood-serum may agglutinate a specific suspension of bacteria in

relatively high titre when the infecting organism is *B. typhosus*, *B. paratyphosus A* or *B* or one of the species of salmonella, e.g. in infections with 'food-poisoning' bacilli or in equine abortion. In these infections, however, the flagellar agglutinin, which is the more obvious, may not only be present as the result of vaccination or a long past infection, but the titre of the patient's serum may be increased by febrile attacks due to other infections, &c. This result of non-specific restimulation of agglutinin formation, known as the anamnætic reaction, has been critically examined and the whole subject reviewed recently by Felix (1929). Flagellar agglutinins, however, appear not to occur in normal serum. The somatic agglutinins formed by the reaction to *B. typhosus*, salmonella and some other bacteria are not exaggerated by non-specific stimuli and are only present in very low titre after vaccination. They are consequently of high diagnostic value, but since somatic agglutinins for many bacteria may be present in very low titre in normal serum, control observations with the bacterial suspension used for the reaction are very necessary. Agglutinins in suspected cases or carriers of cholera are often significant and may be of practical value.

In some infections with non-flagellated bacteria the presence of agglutinins is of great diagnostic importance. Agglutinins for *Br. melitensis*, *Br. abortus*, *B. pullorum*, *B. dysenteriae*, pneumococcus, *B. tularensis* and *B. mallei* are often present in distinct significant amounts during infection. In these and other diseases the diagnosis is often more readily made on clinical grounds or by cultural or other methods. The titre reached by the agglutinins is different for each disease and each kind of bacterium and often for each individual strain of bacterium. It is also different for each patient, but agglutination with the serum may be significant if obtained in dilution varying from 1 in 100 to 1 in 1,000 under different circumstances.

In many species of bacteria, e.g. salmonella, *B. dysenteriae* and *Br. melitensis*, the different serological types render diagnosis by agglutination difficult unless a wide range of cultures is used to prepare the suspensions.

The agglutination of cultures of leptospira is sometimes of value in the diagnosis of haemorrhagic jaundice.

#### GENERAL IMPORTANCE OF THE AGGLUTINATION REACTION : RELATION OF AGGLUTINOGENS TO VIRULENCE AND TO IMMUNIZING CAPACITY.

Agglutination has always been of great importance in the study of immunity on account of the comparative ease and regularity with which agglutination experiments can be carried out *in vitro* and the content of the agglutinins in the serum can be measured. It has, however, acquired fresh importance since the work of Griffith, Dochez, Heidelberger, Avery, Reimann and others on the pneumococcus has shown that virulent cultures can be distinguished from non-virulent by agglutination. This parallelism between pathogenicity and the possession of certain

agglutinogens has also been shown to apply to some extent to salmonella (Topley and Ayrton, 1924; Goyle, 1926<sup>2</sup>; White, 1929) and to *B. typhosus* (Arkwright, 1927<sup>2</sup>), and undoubtedly, but with some reserve in its application, to streptococci (Cowan, 1923; Todd and Lancefield, 1928; Andrewes, 1928; Eagles, 1928; Lancefield and Todd, 1928).

Further evidence of agglutinability as an indication of potential pathogenicity has been brought forward by Weil and Felix (1920), Weil (1921), Felix (1924), Braun and Nodake (1924), Hofmeier (1927), Jones, (1927<sup>3</sup>), Felix and Olitzki (1926), Lubinski (1928), Felix and Robertson (1928), who have shown that a somatic agglutinin is closely correlated if not identical with the precipitating, bactericidal, complement-fixing and opsonic antibodies in immune serum. Complement fixation also apparently occurs to a much less degree with flagellar antigen and antibody (Hofmeier 1927; Balteanu, 1926; Jones, 1928). Felix (1924) has also shown that there is a close connection between the existence of a natural infection and the production of O (somatic) agglutinins for *B. typhosus*. He moreover believes that a prognosis in typhoid fever may often be made from the titre of the O agglutinins in the patient's serum.

That the reaction of the animal body to the smooth S somatic antigen is related to resistance to infection is also indicated by the results obtained by Arkwright (1927<sup>2</sup>) who found that vaccinating guinea-pigs with the non-flagellated smooth form of *B. typhosus* or *B. paratyphosus A* protected them against lethal intraperitoneal inoculation. The comparatively slight effect of the flagellar antigen and the inadequacy of the R somatic antigen was shown by the inefficacy of a vaccine made with the R motile form and also by showing that active antigen in the vaccine was heat-stable (100° C.). Ibrahim and Schütze (1928) found R vaccine of *B. aertrycke* ineffective and obtained good protection with a flagellated S vaccine, but only found slight resistance after this vaccine had been heated at 100° C. for 2 hours. Springut (1927) protected mice against living cultures of 'mouse-typhoid' with a vaccine consisting of H + O antigen, but not with the same vaccine which had been steamed for two hours. Topley (1929) protected mice against natural infection with *B. aertrycke* by means of a S vaccine which had been treated with alcohol to destroy the flagellar antigen and found flagellated R vaccine quite useless.

Schütze (1930) demonstrated the efficacy as a vaccine of the 'smooth' somatic antigen of *B. aertrycke* and *B. enteritidis* Gaertner, which had been heated at 100° C. for 15 minutes, but found the value considerably impaired after two hours at that temperature.

The protection afforded by vaccines of *Pneumococcus* and *Streptococcus* with special agglutination reactions has been shown by Avery, Reimann, Todd, &c. Tillett (1928) has brought forward evidence that by repeated inoculations with R emulsions of pneumococcus an immunity to S cultures of all three types of this organism can be obtained. Julianelle and Avery (1928) have reached similar results especially by intracutaneous inoculation with S or R strains of pneumococcus.

The belief held at one time that agglutinins were no indication of immunity was probably largely due to the fact that till the last fifteen years the flagellar agglutination with serum was in very many cases alone observed, especially in enteric fever and in research with *B. typhosus*, and in these instances the somatic agglutination was entirely ignored.

Bull (1914-16) claimed to have shown that agglutination of several different pathogenic bacteria took place very rapidly in the circulation during life, and maintained that both by itself and in association with phagocytosis, it was largely responsible for the defence in immunity.

To what extent the clumping of bacteria in the animal body is of itself a means of resistance to infection cannot be regarded as settled, but it seems not unlikely that agglutination when it occurs, as it does, after peritoneal inoculations, may facilitate phagocytosis and localization of the infection.

It seems probable that the value of agglutination as an indication of virulence or of immunity is due to the existence of a direct antagonism between the special chemical components of the virulent strain and the specific antibodies rather than to the actual occurrence of clumping. Agglutinability does not appear to be directly related to toxin production.

#### AGGLUTINATION AS A GUIDE TO CLASSIFICATION.

Agglutination has been found, within limits, to be a most valuable aid to the recognition and classification of bacteria which are pathogenic for animals, and to some extent for the differentiation of species in plants and the soil whether as saprophytes or parasites, but in the department of plant bacteriology it has not been very much used (Brooks, Nain and Rhodes, 1925 ; Lacey, 1926). It has been found difficult to classify certain semi-parasitic classes of bacteria such as *B. coli* and staphylococci. This is generally assumed to be due to the multiplicity of species, but it may perhaps be partly explained by their relatively imperfect state of parasitism, and parasitic differentiation.

In bacteria associated with human and animal diseases it has long been known that changes often occur in artificial culture which diminish or increase the pathogenicity. A decline in virulence is known to be often associated with changes in a culture which are recognizable *in vitro*. In old laboratory cultures or under certain other conditions 'spontaneous agglutinability' often appears together with lowered virulence. It is now recognized that both diminished stability of broth cultures or emulsions and lessened pathogenicity are often associated with a change in the chemical nature of the antigenic constituents of the bacilli. It is especially the smooth somatic agglutinogen in the typhoid-colic group and the capsular antigen in *Pneumococcus* which appears to be necessary for virulence.

It is not, at present, clear how far such a change in virulence, which is associated with antigenic change, should be regarded as a temporary loss of antigen by a 'normally' parasitic organism which has become

' non-virulent ', or rather, in the case of enhanced virulence, as an acquirement of new factors and properties by bacteria which have previously lived ' naturally ' as saprophytes. It may be that the change is equally liable to occur in either direction in special instances. For purposes of the present discussion this point may best be disregarded and the forms or variants which are best endowed with distinctive antigens be taken as characteristic of the ' normal ' race. These will be represented by the virulent strains recently derived from the animal body.

If use is made of agglutination for classification, both flagellar and somatic antigens demand consideration, although the two have different relative importance in different families. In *B. proteus* the flagellar antigen only serves to distinguish and limit large groups and the somatic antigen is far more specialized. In *Salmonella*, on the other hand, some of the somatic antigens are very widely distributed in the group and the flagellar agglutination is often needed for making finer distinctions (Weil and Felix, 1920 ; White, 1926). White (1929) showed that the *B. typhosus-coli-dysenteriae* group could be divided by the agglutination reactions of the somatic antigen of the R form and he suggested this method as a useful guide in classification of bacteria into large groups which could be differentiated further by the somatic agglutination of the S form, and by flagellar agglutination.

Schütze (1928) has shown that whereas strains of *B. pseudotuberculosis rodentium* possess quite uniform flagellar antigen, they can be divided into two subgroups by analysis of their somatic antigens. He also showed that certain *Salmonella* strains and the members of one of the subgroups of *B. pseudotuberculosis* were alike in all giving the same serum reactions with their somatic antigen, though in respect of their flagellar antigen the two ' species ' were quite distinct.

*B. mallei* and *B. whitmori* (Stanton and Fletcher, 1925) are another instance of two strains or ' species ' which differ considerably culturally, but possess antigens in common. This connecting antigen is presumably somatic, since only one of the species, *B. whitmori*, is motile. They further resemble each other closely in the morbid conditions which they produce. Their relationship may perhaps be compared to that of the H and O (motile and non-motile) forms of *B. typhosus*, &c., though it cannot be nearly so intimate. The resemblance of the motile *B. pseudotuberculosis rodentium* to the non-motile *B. pestis* is also somewhat similar. In families of bacteria where nothing corresponding to the H or flagellar antigen is known cross-relationships between ' species ' for long believed to have little in common and showing great divergence in their pathological performances have been very fully described from time to time. The *Meningococcus*, though always producing apparently the same disease, can be divided into a number of types by agglutination and absorption techniques (Arkwright, 1909 ; Gordon and Murray, 1915 ; Griffith, 1916 ; Murray, 1929). In the same way types of *Gonococcus* have been described (Torrey, 1907). These two species are often quite indistinguishable by

agglutination and absorption technique. Certain strains of each appear to have identical antigens, i.e. to belong to the same subgroup. Other instances of so-called 'heterogenetic specificity' are known in bacteria. Strains of *Pneumococcus* can be classified by the agglutination of their capsular substance into distinct types which are also separable to some extent by their pathogenic characters. Nevertheless, one of these capsular substances has been found by Heidelberger, Goebel and Avery (1925) to characterize chemically and serologically not only one of the types of *Pneumococcus* but also a type of *B. pneumoniae*, and the corresponding types of the two species cross-agglutinate. Sugg and Neill (1929) found that with a strain of yeast antibodies could be obtained in the horse which agglutinated *Pneumococcus* Type II and gave passive protection against this type of *Pneumococcus* but not against other types. An instance of the value of classification by agglutination from the point of view of clinical medicine and pathology is seen in the case of *Brucella abortus* and *melitensis*. These two micro-organisms, as shown by Evans (1918), cannot be distinguished by agglutination and absorption; though both can be divided into groups and subgroups, each group is made up of members of both 'species'. Though the two species of *Brucella* are derived from very different sources and usually produce very different pathological states, evidence is accumulating that *Br. abortus* may cause a similar disease in man to *Br. melitensis*. The divergence may, therefore, be in the main a case of adaptation to different species of animals (Evans, 1918; Bevan, 1925; Duncan, 1928; Kristensen, 1927).

If the antigens concerned in virulence, and adaptation, can be acquired and discarded in the course of a few passages *in vivo* and *in vitro* they can scarcely be regarded as a very sound basis for classification on an evolutionary hypothesis. It is chiefly the S somatic and to a less extent the flagellar antigen which are most important in medical serology and these also appear to be most liable to change.

From the point of view of pathology and immunity it is the somatic antigen which appears to be the most important, and the information given concerning this factor or these factors by agglutination is, therefore, of great value in the study of pathogenic bacteria.

#### REFERENCES.

- ANDREWES, F. W., 1922, *J. Path. Bact.*, **25**, 505; 1925, *ibid.*, **28**, 345; 1928, *ibid.*, **31**, 132.
- ANDREWES, F. W. & INMAN, A. C., 1919, *Sp. Rep. Ser. Med. Res. Coun.*, Lond., No. 42.
- ARKWRIGHT, J. A., 1909, *J. Hyg., Camb.*, **9**, 104; 1911, *ibid.*, **11**, 515; 1914<sup>1</sup>, *ibid.*, **14**, 261; 1914<sup>2</sup>, *Z. ImmunForsch.*, **22**, Tl. I, Orig., 396; 1921, *J. Path. Bact.*, **24**, 36; 1927<sup>1</sup>, *Lancet*, Lond., i, 13; 1927<sup>2</sup>, *J. Path. Bact.*, **30**, 345; 1927<sup>3</sup>, *ibid.*, **30**, 566; 1928, *ibid.*, **31**, 665.
- ARRHENIUS, S., 1903, *Z. phys. Chem.*, **48**, 415.
- AVERY, O. T. & GOEBEL, W. F., 1929, *J. Exp. Med.*, **50**, 533.
- BAIL, O., 1901, *Prag. med. Wschr.*, **26**, No. 7, cited from *Zbl. Bakt.*, Abt. I, **30**, 36.
- BAINBRIDGE, F. A., 1909, *J. Path. Bact.*, **13**, 443.
- BALTEANU, I., 1926, *J. Path. Bact.*, **29**, 251.

BECHOLD, H., 1904, *Z. phys. Chem.*, **48**, 385.  
 BEINTKER, 1912, *Klin. Jahrb.*, **26**, 381.  
 BENIASCH, M., 1912, *Z. ImmunForsch.*, **12**, Tl. I, Orig., 268.  
 BEVAN, L. E. W., 1925, *Proc. R. Soc. Med.*, **19** Joint Disc., p. 8.  
 BEYER, H. G. & REAGH, A. L., 1904, *J. Med. Res.*, **12**, 313.  
 BIEN, Z., 1924, *Zbl. Bakt.*, Abt. I, Orig., **93**, 196.\*  
 BIEN, Z. & SONNTAG, F., 1917, *Münch. med. Wschr.*, **64**, 1409.  
 BORDET, J., 1896, *Ann. Inst. Pasteur*, **10**, 193; 1899, *ibid.*, **13**, 225.  
 BORDET, J. & GAY, F. P., 1906, *Ann. Inst. Pasteur*, **20**, 467.  
 BOYCOTT, A. E., 1906, *J. Hyg., Camb.*, **6**, 33.  
 BRAUN, H. & LÖWENSTEIN, P., 1923, *Zbl. Bakt.*, Abt. I, Orig., **91**, 1.  
 BRAUN, H. & NODAKE, R., 1924, *Zbl. Bakt.*, Abt. I, Orig., **92**, 429.  
 BRAUN, H. & SCHAEFFER, H., 1919, *Z. Hyg. InfektKr.*, **89**, 339, 358.  
 BROOKS, R. ST. J., NAIN, K. & RHODES, M., 1925, *J. Path. Bact.*, **28**, 203.  
 BULL, C. G., 1914-16, *J. Exp. Med.*, **20**, 237; **23**, 419; **24**, 25.  
 BURNET, E., 1925, *C.R. Acad. Sci., Paris*, **180**, 2085.  
 CASTELLANI, A., 1902, *Z. Hyg. InfektKr.*, **40**, 1.  
 CHARRIN, A. & ROGER, G. H., 1889, *C.R. Soc. Biol., Paris*, 9 s., i, 667.  
 CHICK, H. & MARTIN, C. J., 1912, *J. Physiol.*, **45**, 261.  
 CORNELIUS, T. T., 1929, *J. Path. Bact.*, **32**, 355.  
 COWAN, M. L., 1923, *Brit. J. Exp. Path.*, **4**, 241.  
 CRAW, J., 1905, *J. Hyg., Camb.*, **5**, 113.  
 DEAN, H. R., 1912, *J. Hyg., Camb.*, **12**, 259.  
 DEFALLE, W., 1902, *Ann. Inst. Pasteur*, **16**, 595.  
 DINEUR, 1898, *Bull. Acad. Méd. Belg.*, **12**, 705.  
 DREYER, G., 1909, *J. Path. Bact.*, **13**, 331.  
 DREYER, G. & JEX-BLAKE, A. J., 1906, *J. Path. Bact.*, **11**, 1.  
 DREYER, G. & WALKER, E. W. A., 1909, *Brit. Med. J.*, i, 151.  
 DREYER, G., WALKER, E. W. A. & GIBSON, A. G., 1915, *Lancet, Lond.*, i, 324.  
 DUNCAN, J. T., 1928, *Trans. Soc. Trop. Med. Hyg., Lond.*, **22**, 269.  
 EAGLES, H., 1928, *Brit. J. Exp. Path.*, **9**, 330.  
 EISENBERG, P. & VOLK, R., 1902, *Z. Hyg. InfektKr.*, **40**, 155.  
 v. EISLER, M. & PORGES, O., 1906, *Zbl. Bakt.*, Abt. I, Orig., **42**, 660.  
 ELSER, W. J. & HUNTOON, F. M., 1909, *J. Med. Res.*, **20**, 373.  
 EVANS, A. C., 1918, *J. Infect. Dis.*, **22**, 580.  
 FALK, I. S., 1928, *The Newer Knowledge of Bacteriology*, E. O. Jordan and I. S. Falk (Univ. Chicago Press), p. 565.  
 FALK, I. S., JACOBSON, M. A. & GUSSIN, H. A., 1925, *J. Infect. Dis.*, **37**, 499.  
 FALK, I. S., JENSEN, L. B. & MILLS, J. H., 1927, *J. Bact.*, **15**, 421.  
 FELIX, A., 1924, *J. Immunol.*, **9**, 115; 1929, *J. Hyg., Camb.*, **28**, 418.  
 FELIX, A. & MITZENMACHER, F., 1918, *Wien. klin. Wschr.*, **31**, 988.  
 FELIX, A. & OLITZKI, L., 1926, *J. Immunol.*, **11**, 31; 1928, *J. Hyg., Camb.*, **28**, 55.  
 FELIX, A. & ROBERTSON, M., 1928, *Brit. J. Exp. Path.*, **9**, 6.  
 FELTON, L. D., 1928, *J. Infect. Dis.*, **42**, 248.  
 FICKER, M., 1903, *Berl. klin. Wschr.*, **40**, 1021; 1913, *Handb. d. path. Mikroorg.*, hrsg. Kolle u. Wassermann, **2**, 193.  
 FLEMING, A., 1928, *Brit. J. Exp. Path.*, **9**, 231.  
 FURTH, J. & LANDSTEINER, K., 1928, *J. Exp. Med.*, **47**, 171.  
 GAEHTGENS, W., 1906, *Münch. med. Wschr.*, **53**, 1351; 1907, *Arb. GesundhAmt., Berl.*, **28**, 226.  
 GARDNER, A. D., 1918, *J. Hyg., Camb.*, **17**, 471; 1920, *Sp. Rep. Ser. Med. Res. Coun., Lond.*, No. 51; 1929, *J. Hyg., Camb.*, **28**, 376.  
 GATES, F. L., 1922, *J. Exp. Med.*, **35**, 63.  
 GOEBEL, W. F. & AVERY, O. T., 1929, *J. Exp. Med.*, **50**, 521.  
 GORDON, M. H. & MURRAY, E. G. D., 1915, *R.A.M.C. Jl.*, **25**, 411.  
 GORDON, M. H. AND OTHERS, 1920, *Sp. Rep. Ser. Med. Res. Coun., Lond.*, No. 50.  
 GOYLE, A. N., 1926<sup>1</sup>, *J. Path. Bact.*, **29**, 149; 1926<sup>2</sup>, *ibid.*, **29**, 365.  
 GRIFFITH, F., 1916, *Rep. Loc. Govt. Bd.*, n.s., **110**.  
 GRUBER, M., 1899, *Münch. med. Wschr.*, **46**, 1329.  
 GRUBER, M. & DURHAM, H. E., 1896, *Münch. med. Wschr.*, **43**, 206.  
 GRUNBAUM, A. S., 1896, *Lancet, Lond.*, ii, 806.

GRUSCHKA, TH., 1920, *Z. ImmunForsch.*, **30**, 208; 1923, *ibid.*, **35**, 97.  
 HEIDELBERGER, M. & AVERY, O. T., 1923, *J. Exp. Med.*, **38**, 73; 1924, *ibid.*, **40**, 301.  
 HEIDELBERGER, M. & KENDALL, F. E., 1929, *J. Exp. Med.*, **50**, 809.  
 HEIDELBERGER, M., GOEBEL, W. F. & AVERY, O. T., 1925, *J. Exp. Med.*, **42**, 701.  
 HENRY, B. S., 1928, *Proc. Soc. Exp. Biol.*, N.Y., **26**, 101.  
 HOFMEIER, K., 1927, *Z. ImmunForsch.*, **50**, 71, 509.  
 IHOHIM, H. M. & SCHÜTZE, H., 1928, *Brit. J. Exp. Path.*, **9**, 353.  
 ISSAEF, B., 1893, *Ann. Inst. Pasteur*, **7**, 260.  
 JONES, F. S., 1927, *J. Exp. Med.*, **48**, 291, 303; 1928, *ibid.*, **47**, 245.  
 JOOS, A., 1901, *Z. Hyg. InfektKv.*, **36**, 422; 1903, *Zbl. Bakt.*, Abt. I, Orig., **33**, 762.  
 JULIANELLE, L. A., 1926, *J. Exp. Med.*, **44**, 113, 683.  
 JULIANELLE, L. A. & AVERY, O. T., 1928, *Proc. Soc. Exp. Biol.*, N.Y., **26**, 224, 226,  
     227.  
 KRAUS, R., 1897<sup>1</sup>, *Wien. klin. Wschr.*, **10**, 431; 1897<sup>2</sup>, *ibid.*, **10**, 737.  
 KRAUS, R. & v. PIRQUET, C. F., 1902, *Zbl. Bakt.*, Abt. I, Orig., **32**, 60.  
 KRISTENSEN, M., 1927, *Ugeskr. Laeg.*, No. 49, cited from *Zbl. Bakt.*, Abt. I, Ref.,  
     **91**, 161.  
 KUHN, P. & WOITHE, 1909, *Med. Klinik.*, **5**, 1631.  
 LACEY, M. S., 1926, *Ann. Appl. Biol.*, **13**, 1.  
 LAIDLAW, P. P. & DUDLEY, H. W., 1925, *Brit. J. Exp. Path.*, **6**, 197.  
 LANCEFIELD, R., 1928, *J. Exp. Med.*, **47**, 469, 481.  
 LANCEFIELD, R. & TODD, E. W., 1928, *J. Exp. Med.*, **48**, 769.  
 LANDSTEINER, K. & LEVINE, P., 1926, *Proc. Soc. Exp. Biol.*, N.Y., **24**, 248; 1927,  
     *J. Exp. Med.*, **48**, 213.  
 LEDINGHAM, J. C. G. & DEAN, H. R., 1912, *J. Hyg., Camb.*, **12**, 152.  
 LENTZ, O., 1913, *Handb. d. path. Mikroorg.*, hrsg. Kolle u. Wassermann, **3**, 899.  
 v. LINGELSHEIM, 1913, *Zbl. Bakt.*, Abt. I, Orig., **68**, 577.  
 LOEB, J., 1922-3, *J. Gen. Physiol.*, **5**, 395, 505; 1923-4, *ibid.*, **6**, 215.  
 LUBINSKI, M., 1928, *Zbl. Bakt.*, Abt. I, Orig., **108**, 200.  
 MALVOZ, E., 1897, *Ann. Inst. Pasteur*, **11**, 582.  
 MELLON, R. R. & ANDERSON, L. M., 1919, *J. Immunol.*, **4**, 203.  
 MICHAELIS, L., 1911, *Deuts. med. Wschr.*, **37**, 969.  
 MICHAELIS, L. & DAVIDSOHN, H., 1912, *Biochem. Z.*, **47**, 59.  
 MURRAY, E. G. D., 1929, *Sp. Rep. Ser. Med. Res. Coun.*, Lond., No. **124**, 97.  
 NICOLLE, C., 1898, *C.R. Soc. Biol.*, Paris, **50**, 1054.  
 NICOLLE, C. & TRENEL, M., 1902, *Ann. Inst. Pasteur*, **16**, 562.  
 NICOLLE, M., JOUAN, C. & DEBAINS, E., 1919, *Ann. Inst. Pasteur*, **33**, 261.  
 NORTHROP, J. H., 1928, *The Newer Knowledge of Bacteriology*, E. O. Jordan and  
     I. S. Falk (Univ. Chicago Press), p. 782.  
 NORTHROP, J. H. & DE KUIF, P. H., 1921-2, *J. Gen. Physiol.*, **4**, 655; 1922-3,  
     *ibid.*, **5**, 127, 139.  
 OLITZKI, L., 1928, *Zbl. Bakt.*, Abt. I, Orig., **108**, 247.  
 ORCUTT, M., 1924, *J. Exp. Med.*, **40**, 43, 627.  
 PALTAUF, R., 1897, *Wien. klin. Wschr.*, **10**, 537.  
 PFAUNDLER, M., 1898, *Zbl. Bakt.*, Abt. I, **28**, 71.  
 PFEIFFER, R., 1896, *Zbl. Bakt.*, Abt. I, **19**, 593.  
 PICK, E. P., 1913, *Handb. d. path. Mikroorg.*, hrsg. Kolle u. Wassermann, **1**, 685.  
 PIJPER, A., 1923, *S. Afr. Med. Rec.*, **21**, 51, 74.  
 PIJPER, A. & DAW, H., 1930, *Brit. J. Exp. Path.*, **11**, 112.  
 PIJPER, A. & PULLINGER, B. D., 1928, *Brit. Med. J.*, i, 587.  
 PORGES, O., 1905, *Z. Exp. Path. Ther.*, **1**, 621.  
 PORGES, O. & PRANTSCHOFF, A., 1906, *Zbl. Bakt.*, Abt. I, Orig., **41**, 466, 546, 658.  
 PUTTER, E. & ZORN, W., 1924, *Z. ImmunForsch.*, **39**, 469.  
 REIMANN, H. A., 1926, *J. Exp. Med.*, **48**, 107.  
 RONA, P. & MICHAELIS, L., 1910, *Biochem. Z.*, **28**, 193.  
 SACHS, H., 1917, *Deuts. med. Wschr.*, **43**, 964.  
 SAVAGE, W. G., 1901, *J. Path. Bact.*, **7**, 388.  
 SCHIFF, F., 1922, *Z. ImmunForsch.*, Tl. I, Orig., **33**, 511; 1925, *Handb. d. Biochemie  
     d. Menschen u. d. Tiere*, hrsg. C. Oppenheimer, Jena, **3**, 262.  
 SCHÜTZE, H., 1920, *Lancet*, Lond., i, 93; 1922, *J. Hyg., Camb.*, **20**, 330; 1928,  
     *Arch. Hyg.*, Berl., **100**, 181; 1930, *Brit. J. Exp. Path.*, **11**, 34.

SHIBLEY, G. S., 1924, *J. Exp. Med.*, **39**, 245 and **40**, 453; 1925, *Proc. Soc. Exp. Biol.*, N.Y., **22**, 276; 1926, *J. Exp. Med.*, **44**, 667; 1929, *ibid.*, **50**, 825.

SMITH, TH. & REAGH, A. L., 1903, *J. Med. Res.*, **10**, 89.

SMITH, TH. & TENBROECK, C., 1915, *J. Med. Res.*, **31**, 503, 547.

SPRINGUT, E., 1927, *Z. ImmunForsch.*, **52**, 25.

STANTON, A. T. & FLETCHER, W., 1925, *J. Hyg.*, Camb., **23**, 347.

STUART, G. & KRIKORIAN, K. S., 1928, *J. Hyg.*, Camb., **28**, 105.

SUGG, J. Y. & NEILL, J. M., 1929, *J. Exp. Med.*, **49**, 183.

TILLETT, W. S., 1928, *J. Exp. Med.*, **48**, 791.

TODD, E. W., 1928, *Brit. J. Exp. Path.*, **9**, 1.

TODD, E. W. & LANCEFIELD, R., 1928, *J. Exp. Med.*, **48**, 751.

TOPLEY, W. W. C., 1929, *Lancet*, Lond., **i**, 1337.

TOPLEY, W. W. C. & AYRTON, J., 1924, *J. Hyg.*, Camb., **22**, 222.

TOPLEY, W. W. C. & PLATTS, S. G., 1918, *Lancet*, Lond., **i**, 800.

TOPLEY, W. W. C. & WILSON, G. S., 1929, *The Principles of Bacteriology and Immunity*, London, **1**, 188.

TOPLEY, W. W. C., GREENWOOD, M., WILSON, J. & NEWBOLD, E., 1929, Communication to Path. Soc. G. Brit. and Ireland, Jan., 1929.

TORREY, J. C., 1907, *J. Med. Res.*, **16**, 329.

WALKER, E. W. AINLEY, 1922, *Proc. Roy. Soc.*, B, **93**, 54.

WASHBOURN, J. W., 1895, *J. Path. Bact.*, **3**, 214.

WEIL, E., 1905, *Arch. Hyg.*, Berl., **53**, 291; 1911, *Biochem. Z.*, **33**, 56; 1921, *Z. ImmunForsch.*, Tl. I, Orig., **31**, 50.

WEIL, E. & FELIX, A., 1916, *Wien. klin. Wschr.*, **29**, 33; 1917, *ibid.*, **30**, 1509; 1918, *ibid.*, **31**, 637; 1920, *Z. ImmunForsch.*, Tl. I, Orig., **29**, 24.

WEIL, E., FELIX, A. & MITZENMACHER, F., 1918, *Wien. klin. Wschr.*, **31**, 1226.

WHITE, P. B., 1925, *Sp. Rep. Ser. Med. Res. Coun.*, Lond., No. **91**, 74; 1926, *ibid.*, **103**, 50; 1928, *J. Path. Bact.*, **31**, 423; 1929, *ibid.*, **32**, 85.

WIDAL, F., 1896<sup>1</sup>, *Bull. Soc. med. Hôp. Paris*, 3 s., **13**; 1896<sup>2</sup>, *J. Méd. Chir. prat.*, 533.

WIDAL, F. & SICARD, M. A., 1897, *Ann. Inst. Pasteur*, **11**, 353.

WOLLSTEIN, M., 1907, *J. Exp. Med.*, **9**, 588.

ZINSSER, H. & PARKER, J. T., 1923, *J. Exp. Med.*, **37**, 275.

## CHAPTER XIII. THE PRECIPITATION REACTION.

By H. R. DEAN (UNIVERSITY OF CAMBRIDGE).

### Introduction.

AT a session of the K.K. Gesellschaft der Aertzte in Vienna on April 30th, 1897, Rudolf Kraus (1897<sup>1</sup>) made the first communication on the precipitation reaction. A culture of the cholera vibrio was filtered and the filtrate was tested for sterility. To the bacteria-free clear filtrate was added anticholera serum. After some time at 37° C. a cloudiness and then flocculi appeared in the mixture. After 24 hours there was a finely flocculent precipitate and a clear supernatant fluid. A similar precipitate formed in a mixture of antityphoid serum with a filtered culture of *B. typhosus*. When typhoid filtrate was mixed with anticholera serum or antityphoid serum with cholera filtrate the mixtures remained clear. The reaction which resulted in the formation of a precipitate was specific. Later in the same year Kraus (1897<sup>2</sup>) published a more detailed account of his experiments. The discovery of Kraus was confirmed by Nicolle (1898) and other workers. In 1899 Tchistovitch (1899) injected eel-serum into rabbits, guinea-pigs, dogs and goats and obtained antisera which formed a precipitate when mixed with eel-serum. In the same year Bordet (1899) prepared an antiserum by injecting milk into rabbits. A mixture of this antiserum with milk showed the formation of granules and flocculi. In 1900 Myers (1900) injected crystallized egg-albumin, prepared by the method of Hopkins and Pinkus, into the peritoneal cavity of rabbits and obtained an antiserum which formed a precipitate when mixed with crystallized egg-albumin. Kowarski (1901) immunized rabbits with an extract of wheat-flour. The resulting antiserum formed a precipitate when mixed with wheat-flour. Within a few years of the publication of Kraus's discovery precipitating antisera had been prepared against a great variety of antigens of animal, vegetable and bacterial origin. For a review of the literature on the precipitation reaction to May 1911 the reader is referred to Kraus's article in Kolle and Wassermann's *Handbuch der pathogenen Mikroorganismen*, second edition, Vol. II, Part I, p. 732.

Kraus named the substance present in the bacterial filtrate precipitinogen while the term precipitin was used to denote the hypothetical substance or antibody formed or set free in the animal body in response to injections of an antigen or precipitinogen. It was generally assumed at first that a specific substance present in the antiserum, precipitin, was the active agent which precipitated or coagulated the other ingredient, the precipitinogen. In due course the precipitin took rank in Ehrlich's

nomenclature as an antibody or receptor of the second order, complete with haptophore and ergophore groups. The words precipitin and precipitinogen have passed into the everyday jargon of the laboratory. We are unable to teach, we can hardly describe the results of experiments without using these and similar words. They have served as pegs on which to hang our thoughts and theories, and until we have more knowledge of what happens in these wonderfully specific reactions which take place in a mixture of the serum of an inoculated animal with the substance which has been inoculated, it is unlikely that we shall wish to coin new words to replace them. Nevertheless, it is well to remember that we have no direct evidence of any such substance as a precipitin, and we have no conclusive evidence that antibodies, in the sense of chemical substances, exist. We know that when certain complex substances, which we call antigens, are introduced into the body of an animal in the course of disease or by a syringe, the blood and tissues are so altered that a reaction occurs when the altered tissues or blood fluids are brought into contact with a substance the same or very similar to that which has been injected. Under certain experimental conditions this reaction results in the formation of a precipitate.

### **Preparation of Precipitating Sera.**

In general relatively large doses and a relatively long series of injections are required. Rabbits are almost invariably used. In our experience better results are obtained if at least two series of injections are given. Six to eight injections, each of 2 c.cm., of alien serum are given at five-day intervals. After a rest of 3 to 6 months a second series of injections is given. The first few injections may be intravenous, the subsequent injections should be intraperitoneal. Details of the preparation of precipitating sera have been given by Fornet and Müller (1910), Bonhoff and Tsuzuki (1910), Tsuzuki (1910), Dean and Webb (1926, 1928), Hitchcock (1924) and Todd and Lancefield (1928).

### **Methods of Performing the Precipitation Reaction.**

Wassermann and Schütze (Kraus, 1912) used a constant quantity of the antigen-containing substance and falling quantities of the antiserum. Uhlenhuth (Kraus, 1912) kept the antiserum volume constant and varied the quantities of the antigen. Dilutions of horse-serum from 1 in 100 to 1 in 1,000,000 are prepared. Each dilution is contained in a volume of 1 c.cm. To every tube 1 c.cm. of antiserum is added. Precipitation is allowed to proceed for a certain time at a certain temperature, e.g. 1 hour at 55° C., 4 hours at 37° C., or 24 hours at room temperature. The titre of the antiserum is expressed as the greatest dilution of horse-serum which produces a definite turbidity. In Fornet and Müller's (1910) ring test constant volumes of the antiserum are placed in a series of tubes. Progressive dilutions of the antigen, e.g. horse-serum, are prepared and

cautiously introduced drop by drop on the wall of the test-tube so as to form a separate layer on the surface of the antiserum. The highest dilution of the antigen-containing fluid which produces a definite ring is taken as the titre of the antiserum. The optimal proportions method of titration is described below.

### The Influence of Optimal Proportions of Antigen and Antibody in the Reaction.

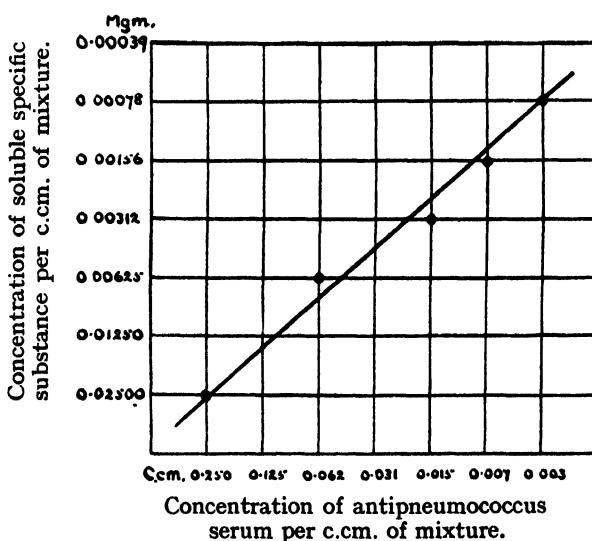
Of the factors which may influence the formation of a precipitate the relative proportions in which antigen and antibody are present in the mixture is one of the most important. Danysz (1902) showed that ricin and antiricin must be mixed in optimal proportions for the formation of the most voluminous precipitate and for the complete neutralization of the poison. Calmette and Massol (1909) mixed a series of quantities of antivenom serum with a constant amount of cobra venom and found that the best flocculation took place at the neutral point determined by animal inoculation and that the precipitation reaction could be used for the approximate measurement of the antitoxin content of a serum. Dean (1911<sup>1 & 2</sup>) showed that the amount of precipitate depends on the relative proportions of antigen and antibody present in the mixture. With a 1 in 5 dilution of the antiserum the largest precipitate was obtained by addition of a 1 in 8 dilution of the antigen (horse-serum). With half of the quantity of antiserum, 1 in 10 dilution, the largest precipitate was obtained with a 1 in 16 dilution of the antigen. Similar results were obtained in mixtures of extracts of typhoid bacilli with an antityphoid serum. If a series of quantities of antigen were mixed with a constant amount of antiserum it was found that for any given quantity of antiserum there existed a quantity of antigen which was able to produce the largest precipitate. If to a duplicate series of amounts of antigen there was added half the amount of antiserum, the largest precipitate was found to be about half the largest precipitate of the first series and was found in the tube which contained half the amount of antigen which produced the largest precipitate in the first series. When either antigen or antibody was present in relative excess the reaction was delayed. Nicolle, Debains and Césari (1919) described a method for the titration of horse- and antihorse-serum, of diphtheria and tetanus toxin and antitoxin. The toxin mixed with neutral gelatin was run into a series of tubes and the mixture was allowed to set. Serial dilutions of antitoxin were added to the tubes containing the gelatin toxin mixture and the tube which showed the best ring formation at the junction was observed. The result obtained was confirmed by animal experiment. In a series of papers published between 1922 and 1924 Ramon (1922, &c.) has described the flocculation method for the titration of diphtheria toxin and antitoxin which is named after him. The Ramon reaction is an excellent example of the great importance of the proportions in which antigen and antibody are mixed in the precipitation reaction. The technique of the Ramon reaction is described

in detail in papers published by Glenny and Okell (1924), by Glenny, Pope and Waddington (1925) and by Glenny and Wallace (1925).

The quantitative relationship of antigen to antibody was studied by Opie (1923), who used horse-serum, donkey-serum and crystalline egg-albumin together with antisera prepared by the injection of these antigens into rabbits. Opie found that a precipitate might be dissolved by relative excess of antigen, and attributed the inhibition of precipitation in the zone of antigen excess to the solvent action of excess of antigen. Opie stated that when a constant quantity of antigen is mixed with increasing quantities of immune serum the maximum amount of precipitate is found when antigen is mixed with several hundred times its volume of immune serum, the number of volumes depending upon the strength of the anti-serum. This relation furnished a measure of the strength of the antiserum in multiples of the volume of antigen used for the test.

Parker (1923) employed Zinsser and Parker's 'residue' antigens, bacterial products which did not give the well-known chemical tests for protein, and was able to confirm the importance of optimal proportions of antigen and antibody in the serum reactions. Similar results were obtained by Morgan (1923), who employed the 'soluble specific substance' of the pneumococcus and antipneumococcus serum. Chart I taken from Morgan's paper illustrates the results obtained when various quantities of antiserum were mixed with various quantities of the soluble specific substance.

CHART I.



In this chart the ordinates represent, in milligrammes, the concentration of soluble specific substance, and the abscissæ represent, in cubic centimetres, the concentration of immune serum. The points on the chart which correspond with the maximum precipitate for any concentration of the antiserum fall approximately on a straight line. Both

Parker and Morgan confirmed the importance of the relative proportions of antigen and antibody in the serum reactions but their results have, as Morgan pointed out, additional interest from the fact that the bacterial substances which they mixed with antiserum, 'the residue antigens' and 'soluble specific substances' were protein-free. The inhibition or solution of the precipitate, in their experiments, in the zone of relative antigen excess is evidence that this inhibition or solution is due to excess of the actual specific substance and not to the presence of an indifferent protein substance acting as a protective colloid. Dean and Webb (1926) elaborated an optimal proportions method for the quantitative determination of either antigen or antibody and applied this method to the determination of the rate of antibody production in rabbits after an injection of horse-serum. Tomcsik (1927) found that the principle of optimal proportions held good in mixtures of an antiserum with the carbohydrate hapten obtained from a yeast. Goldsworthy (1928) drew attention to the occurrence of multiple zones of particulation in mixtures of horse-serum with certain specimens of antiserum.

#### **Titration of Antiserum by the Optimal Proportions Method.**

The titre is determined in two stages: (1) by a rough test giving approximate results, (2) by a fine test. In the rough test a series of dilutions of the horse-serum are tested with a series of dilutions of antiserum. Four dilutions of antiserum, 1 in 5, 1 in 10, 1 in 20 and 1 in 40, and eleven dilutions of the antigen from 1 in 10 to 1 in 10,000 are usually sufficient. Such an experiment gives a rough estimate of the relative proportions in which the two ingredients should be mixed in order to produce a particulate precipitate. Sufficient information can often be obtained if a series of antigen dilutions is titrated with one dilution of antiserum only. The use of a range of antiserum dilutions does, however, afford valuable information. An idea is formed as to the highest dilution of antiserum which can be used with satisfactory results. If the antiserum is to be used for the differentiation of the homologous serum from the serum of nearly allied species it is important to use the antiserum in the highest dilution which will yield visible precipitates when mixed with dilutions of the homologous protein. Again the speed of the reaction depends on the strength of the particular antiserum and on the concentration of the antiserum in the mixture. The greater the dilution the slower is the reaction. If it is proposed to determine accurately the proportions of antigen and antibody most favourable for the formation of particles it is desirable to select a dilution of the antiserum the use of which will ensure the appearance of particles within a reasonable time. But if the antiserum is too concentrated flocculation may occur so rapidly that it is impossible to pick out the tube in which the first particles appear.

The optimal proportions are exactly determined by a fine test, which is set up as follows. A 1 in 100 dilution of horse-serum, the antigen, is prepared in a 100 c.cm. volumetric flask. From this 1 in 100 dilution a

further dilution is prepared, say 1 in 400, in accord with the indications of the rough test. Ten perfectly clean tubes of similar make and diameter are set out and numbered. Of the 1 in 400 dilutions quantities falling from 1 c.cm. to 0·1 c.cm. are delivered into each of the ten tubes. The difference between each tube is thus 0·00025 c.cm. of horse-serum. The volume in each tube is made up to 1 c.cm. by adding an appropriate volume of saline solution. To every tube is added 1 c.cm. of an appropriate dilution of antiserum, say 1 in 40. The volume of antiserum in each tube is 0·025 c.cm. The rack containing the tubes is kept at room temperature and under constant observation. Good illumination is necessary and the progress of events is observed against a dull black background illuminated by a shielded electric light. The formation of particles is watched with the naked eye and with a reading-glass. The period chosen for the reading is that during which particles are first visible. In many cases it is possible to detect the tube in which particles first appear and there may be a short interval of time during which particles are present in one tube and in one tube only. But as a rule particulation occurs in two or three tubes in quick succession, and, as several tubes have to be kept under observation, it is often difficult to be sure of the tube in which particles first appear. But after particulation has occurred in the leading two or three, or even four tubes, there is as a rule a considerable period during which the particles are distinctly and unquestionably larger in one tube of the series than they are in any other tube. It is this period which is chosen for making the decisive reading.

Suitable dilutions or quantities of both antiserum and antigen must be determined by preliminary experiments. Of the antiserum dilutions, 1 in 10, 1 in 20 or 1 in 40 are usually suitable. In the majority of experiments a 1 in 20 dilution has been used. Strong or quickly acting antisera can be used with advantage in a 1 in 40 dilution. The proportions of antigen and antibody need adjustment so that the critical tubes come in the centre of the rack. This is effected most conveniently by adjusting the concentration of antigen in the stock antigen dilution. When, as occasionally happens, two tubes run a dead heat a decision can be obtained by altering the strength of the antigen dilution which is used.

By this method it is possible to distinguish quantitative differences as small as 0·0001 c.cm. of horse-serum. In the case of a very strong precipitating antiserum the antigen-antiserum ratio may be about 1 to 10, that is to say that one volume of the particular antiserum flocculates most quickly with a tenth of its volume of the antigen. A weaker antiserum may have an antigen-antiserum ratio of 1 to 100. Details of the titration of antiserum by the optimal proportions method are given by Dean and Webb (1926, 1928).

If the proportions which favour the most rapid formation of particles can be regarded as the proportions in which the antibody is neutralized by the antigen it follows that the larger the proportion of the antigen in the mixture in which the earliest particulation occurs the larger is the antibody

content of the antiserum. That is to say, the optimal proportions method may be employed for the quantitative determination of either antigen or antibody. The antigen-antiserum ratio with an antiserum rich in antibody will be a relatively low figure and with a weak antiserum a relatively high figure.

If the ratio figure depends on the antibody content of the serum it becomes possible to express the antibody content in units. For this purpose Dean and Webb (1926, 1928) have made the assumption that a unit of antibody is contained in the volume of an antiserum which particulates most rapidly with 0·00001 c.cm. of normal horse-serum. If the volume of antiserum which particulated most rapidly with 0·00001 c.cm. of horse-serum were 1 c.cm., such an antiserum would be said to contain one unit of antibody per c.cm. It would be in practice too weak to permit of titration by the optimal proportion method, but, in order to provide a convenient notation, 0·00001 c.cm. is suggested as the amount of horse-serum which reacts with one unit of antibody. For example, a specimen of antiserum was found to have an antigen-antiserum ratio of 1 to 200. Therefore 0·00001 c.cm. of horse-serum would react with two hundred times its volume, or 0·002 c.cm. of antiserum. That is to say, 1 unit of antibody was contained in 0·002 c.cm. of antiserum and 500 units in 1 c.cm.

### The Influence of Reaction, Temperature, Salts and Dilution.

Michaelis and Davidsohn (1912) found that agglutination and precipitation reactions can occur within a considerable range of H-ion concentration, and that the optimum reaction did not correspond with the isoelectric point of either the antigen or the serum globulin of the antibody. Only in the case of very dilute antigen or antibody solutions does the pH have much influence and then the results are best at about the neutral point. The subject has been reinvestigated by Mason (1922), who criticized Michaelis and Davidsohn for the use of sodium acetate and acetic acid mixtures as these are liable to cause non-specific protein precipitation. Mason employed sodium hydrate and phosphoric acid mixtures. He found that precipitation occurred between pH 4·5 and pH 9·5. Precipitates already formed dissolved in mixtures more alkaline than pH 9·5 or more acid than pH 4·5.

The effect of temperature on the reaction is well marked. The reaction may be performed at any temperature from 0 to 58° C. Precipitation occurs much more rapidly at 37° C. than at room temperature and still more rapidly at a temperature of 55° C. At temperatures between 0° C. and 5° C. the reaction is extremely slow. The influence of various concentrations of sodium chloride on the reaction has been investigated by many workers and there is not a little difference of opinion. So-called physiological saline solution, 0·85 per cent. solution of sodium chloride in distilled water, is the usual diluent. In the precipitation reaction the antiserum is commonly used undiluted or in dilutions of 1 in 10 to 1 in 40. In mixtures in which the antiserum is diluted to 1 in 100 or more, the

reaction is greatly delayed, and if the precipitate is visible at all it makes its appearance as a diffuse turbidity which settles very slowly to the bottom of the tube. The precipitate is no doubt dispersed by the relatively large volume of sodium chloride solution. The writer and R. A. Webb have carried out a considerable series of experiments hitherto unpublished. Solutions of sodium chloride in distilled water from 0·01 to 2 per cent. were prepared. It was found that precipitation reactions could be carried out in sodium chloride solutions of strengths from 0·02 to 1 per cent., and that within this wide range the ratio of antigen to antiserum, i.e. the optimal proportions of antigen to antiserum for flocculation, did not vary. The speed of the reaction and the time at which particles were first visible was affected by the concentration of salt. The speed steadily increased from 1 to 0·2 per cent. and the conclusion was that, at any rate within the range of serum and antiserum dilutions which were used, 0·2 per cent. was the most favourable concentration of sodium chloride for the precipitation reaction. With concentrations of salt above 1 per cent. the reaction was slower and the ratio in which antigen reacted with antiserum was altered.

Doerr (1920) has investigated the influence of sodium chloride and other salts on the precipitation reaction. In mixtures of human serum and antihuman serum in the presence of sufficient electrolyte a portion of the globulin passes from the sol to the gel state. The union of precipitin and precipitinogen results in a loss of stability and an increased susceptibility to salt. The formation of the precipitate is hindered by excess of either antigen or antiserum and this interference with precipitation by antigen excess is specific. The precipitate washed in 1 per cent. sodium chloride and resuspended may be dissolved by the addition of adequate quantities of the antigen-containing serum. According to this view precipitation is a reversible reaction and the globulin gel may be reconverted into a globulin sol. The quantity of the antigen serum necessary to dissolve the precipitate depended on the proportions of antigen and antiserum which had been used to produce the precipitate.

If normal serum is mixed with a solution of thorium sulphate in certain proportions, a precipitate results. The precipitate does not form in the presence of excess of either ingredient and already formed precipitates may be dissolved by either ingredient. For the solution of the precipitate any normal serum may be used. Moreover the specific precipitates which are formed by the interaction of antigen and antiserum may be redissolved by thorium sulphate. If a precipitate is formed by mixing thorium sulphate with a normal serum and the precipitate is redissolved in thorium sulphate the resulting solution retains the antigen specificity of the original serum. Preparations of this kind have been used to sensitize and to produce anaphylactic shock in guinea-pigs. A weak immune serum may be precipitated with thorium sulphate and the resulting precipitate may be redissolved in another immune serum with a resulting increase in antibody concentration.

### Source of the Precipitate.

The name precipitin given to a hypothetical substance present in a precipitating antiserum implied the view that the antiserum precipitated the homologous protein. The analogy with the agglutination reaction was tempting and Bordet and Gengou (1901<sup>1</sup>) state specifically 'Très généralement, en effet, le sérum d'un animal d'espèce A, injecté de sérum d'espèce B précipite ce sérum B'. On the other hand, evidence that under ordinary experimental conditions the bulk of the precipitate is derived from the proteins of the antiserum has been put forward by many authors. Myers (1900) obtained an antiserum by the injection of Witte's peptone and on mixing this antiserum with a solution of Witte's peptone a precipitate was obtained which did not give a biuret reaction although it gave the other general reactions of proteins. Pick (1902) mixed a bacterial extract which was almost free from protein with the homologous antiserum and obtained a precipitate which contained a considerable mass of protein. Moll (1906) prepared an antiserum against albumin. From the resulting antiserum the globulin fraction was separated. A mixture of albumin, the antigen, with the globulin of the antiserum yielded a precipitate which consisted of globulin. Welsh and Chapman (1906<sup>1&2</sup>, 1910) were able to show that if 2·5 mgm. of dried hen egg-white were mixed with 19 c.cm. antiserum the resulting precipitate had a dry weight of 18 mgm. or more than seven times the weight of the homologous protein concerned. Under the experimental conditions which are customary in precipitation reactions the greater part of the precipitate is derived from the proteins of the antiserum. The evidence on this point is conclusive and the point is of importance not only to an understanding of precipitation but of the whole process of immunity. During immunization the blood-proteins are so altered that their molecules may, under certain experimental conditions, be aggregated and precipitated by the antigen which has been used for immunization.

In 1916 Weil (1916) produced a very pretty proof that both antigen and antiserum are present in the precipitate. Weil mixed 0·02 c.cm. of normal horse-serum with 1 c.cm. of antihorse-serum. The precipitate was collected and washed and divided into four portions which were injected into four guinea-pigs. Four days later two of these animals received 0·5 c.cm. of normal horse-serum. These two animals had been rendered passively anaphylactic by the antihorse-serum contained in the precipitate and both died of anaphylactic shock. At the end of six weeks the other two guinea-pigs were given an injection of 0·5 c.cm. of normal horse-serum and both died of anaphylactic shock. These two animals had been actively sensitized to horse-serum by the horse-serum present in the precipitate. It was proved that the precipitate had contained both antigen and antiserum. Not only had both ingredients of the reaction been contained in the precipitate, but each had been contained in such condition that it could produce the same effect as might have been produced

if it had been separately injected. It seems to be established that under ordinary experimental conditions the precipitate contains both antigen and antiserum, but that the greater part of the mass of the precipitate is derived from the antiserum. The weight of the precipitate and probably its constitution depend on the proportions in which antigen and antiserum are mixed. Nevertheless the proportions of antigen to antibody which are most favourable for rapid particulation are not those which yield, when the reaction is complete, the largest precipitate. If a series of quantities of antigen are set up with a constant quantity of the antiserum it will be found that the weight of the precipitate increases with increase of antigen in the mixture until the tube is reached which contains antigen and antiserum in the proportions most favourable to rapid particulation. In the mixtures containing still more antigen, while flocculation is slower, the ultimate weight of the precipitate is greater until the tube is reached which contains about twice the amount of antigen most favourable to rapid particulation. In tubes which contain more than twice the amount of antigen most favourable to rapid particulation precipitation is very slow, the amount of precipitate is less than the maximum and the supernatant fluid remains opalescent or turbid.

In certain circumstances substances, other than the antigen-containing fluid and the antiserum, which may be added to the mixture, may contribute to the formation of a precipitate. Dean (1912) investigated the effect of adding fresh guinea-pig serum or the euglobulin obtained from a guinea-pig serum to mixtures of an extract of *B. typhosus* with antityphoid serum. If a series of dilutions of an antiserum were prepared a dilution could be selected which when mixed with an appropriate quantity of the homologous antigen formed no precipitate but nevertheless bound complement. If complement was added to such a mixture of antigen and antiserum a precipitate appeared after an interval of 6 to 24 hours. By using an appropriate mixture of antigen and antiserum the amount of the precipitate could be increased, within limits, by increasing the amount of guinea-pig serum in the mixture. Similar results were obtained by the substitution for normal guinea-pig serum of normal rabbit-serum, normal ox-serum or the euglobulin prepared from normal guinea-pig serum. Normal guinea-pig serum which had been heated at 56° C. for half an hour gave similar results but not quite so much additional precipitate as the fresh serum. The precipitates which formed in these mixtures of antigen and antiserum with guinea-pig serum appeared to form more readily at 0° C. In some experiments it was possible to show that precipitates which had formed at 0° C. dissolved when the mixture was placed in an incubator at 37° C. and re-formed when the tubes were restored to the cold chamber. The bearing of these experiments on the mechanism of complement fixation will be discussed in a later section. These additional precipitates were only formed in those tubes which contained antigen and antibody in optimal proportions.

Georgi (1920) added a suspension of heart lipoids with cholesterol to a series of mixtures of toxin and antitoxin. Flocculation occurred in the tubes near the neutral point. Glenny and Okell (1924) have confirmed Georgi's observations. They found that the particles of a cholesterol heart-extract mixture were aggregated and flocculated in mixtures of diphtheria toxin with antitoxin and that the resulting precipitate is more bulky than the precipitate in simple mixtures of toxin and antitoxin. It appears from these experiments that easily precipitable substances may be precipitated from suspensions by mixtures of antigen and antibody in suitable proportions. It is possible that the use of these indicator suspensions may be extended and provide valuable modifications of the precipitation reaction. Nevertheless, it appears improbable that indifferent substances form any considerable part of the precipitate when the reaction is performed under the usual experimental conditions. It seems certain that almost all the precipitate is derived from the globulin of the antiserum.

In the precipitation reaction it seems probable that the globulins are precipitated by the antigen. An antiserum may be regarded as a serum in which the globulin has been so altered that it is precipitated when it is mixed with the homologous protein or antigen. In what this alteration consists represents the great problem of the serum reactions. It is customary to talk about an antiserum as if it was a serum which contained a new and specific chemical substance, an antibody, added to the blood by some cells or tissues charged with the function of antibody formation. A phial of diphtheria antitoxin is regarded as containing so many units of antitoxin, the essential chemical antidote, contained in a vehicle or diluent, the serum-proteins. But all efforts to separate antibodies from the associated globulins have failed. Is there any reason to think any such substance as an antibody exists? We do know that as a result of injections the blood is so altered that the serum becomes precipitable when mixed with the homologous protein. It seems possible that the injected foreign protein may enter into combination or be mixed with the blood-proteins so as to form a complex precipitable in the presence of a further quantity of the same foreign protein. Such a conception implies the persistence of antigen in the blood and the gradual amalgamation of the foreign protein with the globulin of the injected animal to form the altered globulin which is the specific constituent of antiserum. For this point of view some evidence has been put forward by the author (Dean, 1923). Convincing proof of the truth of this hypothesis is, of course, lacking but it demands no greater strain on the imagination than the usually accepted theory of the mode of production of antibodies.

Concerning the content of the supernatant fluid after the completion of the precipitation process various and contradictory statements have been made. Eisenberg (1902) stated categorically that in every precipitation reaction, in addition to the product of the reaction, there persists in the supernatant fluid an excess of both the reagents, which coexist side by side

without reaction. v. Dungern (1903) prepared antisera against the plasma of the octopus and various crustacea and molluscs. He mixed various dilutions of the antigen-containing plasma with a constant volume of the antiserum. The supernatant fluids were separated and tested for the presence of antigen and antiserum. In such a series he always found a middle zone in which the relative concentrations of antibody and antigen were such that both reagents combined quantitatively and went out of solution in the form of a precipitate. In no case did he find both reagents present in the supernatant fluid. Weil mixed horse-serum and antiserum, removed the precipitate with a centrifuge and found that the supernatant fluid contained both antigen and antibody. The same results were obtained with raw egg-albumin and the serum of a rabbit immunized therewith. But if purified crystalline egg-albumin was mixed in various proportions with the serum of a rabbit immunized therewith and if the resulting precipitates were removed, the supernatant fluids contained either antigen or antibody but never both. Bayne-Jones (1917), who used purified protein-antigens, edestin and crystalline egg-albumin, was unable to confirm Weil's results. When a pure protein-antigen was mixed in proper proportions with the serum of a specifically immunized rabbit and the resulting precipitate removed, the supernatant fluid contained both antigen and antibody. Opie (1923) has pointed out that crystalline egg-albumin is an 'almost' pure antigen but contains very small amounts of extraneous antigen capable of forming antibody. Opie thinks that the presence of antigen and antibody in the supernatant fluid is best explained by the assumption of a multiplicity of antigens. On the other hand, Welsh and Chapman (1906<sup>&2</sup>, 1910) stated that when the reaction is complete the whole of the antibody but only a small fraction of the antigen has been removed from solution.

The different results obtained by different workers depend no doubt on differences in experimental methods. The content of the supernatant fluid depends to a great extent on the proportions of antigen and antibody present in the original mixture. Dean and Webb (1926, 1928) found that when antigen and antibody were mixed in proportions most favourable to particulation the antigen disappeared completely or almost completely from the supernatant fluid. In mixtures which contain less than the proportional amount of antigen and a relative excess of antiserum the whole of the antigen disappears. In tubes which contain a relative excess of antigen the disappearance of antigen is difficult to determine because the supernatant fluid remains turbid. Our results indicated that in mixtures containing a relative excess of antigen more than the amount of antigen which was necessary for rapid flocculation was used up. In mixtures containing antigen and antiserum in proportions most favourable to particulation the whole, or practically the whole of the antiserum, disappeared from the supernatant fluid. If antigen and antibody were mixed in proportions most favourable to flocculation practically all the antigen and all the antibody were used up. Mixtures were set up containing

three-quarters, half, one-quarter and one-eighth of the optimal volume of antigen. After the separation of the precipitate the supernatant fluids were found to contain one-quarter, one-half, three-quarters and seven-eighths respectively of the antibody originally present in the mixtures. That is to say in mixtures containing less than the equivalent proportion of antigen the amount of antibody which reacts with antigen is proportional to the amount of antigen in the mixture.

The difficulty of arriving at conclusions as to the end results of the precipitation reaction has been attributed by many authors to the fact that the complex reagents which are commonly used contain a number of antigens and a corresponding number of antibodies. On the other hand, we have been impressed by the remarkably uniform results which we have obtained with various specimens of horse-serum. When the antigen-antibody ratio of a specimen of antihorse-serum has been determined by titration with a specimen of normal horse-serum, the optimal proportions for flocculation have been found to hold good when this antiserum was tested with normal serum obtained from seven different horses. In a series of unpublished experiments R. A. Webb has titrated specimens of normal horse-serum and of diphtheria antitoxin with antisera prepared by the injection of rabbits with normal horse-serum. In other experiments antitoxin and normal horse-serum have been titrated with antiserum prepared by the injection of antitoxin into rabbits. All specimens of both normal and antitoxic horse-serum have reacted in identical proportions with either antisera prepared by injection of the normal serum or antisera prepared by injection of the antitoxin. The process of immunization with diphtheria toxin appears to have no effect on the antigenic content of horse-serum. It does not seem at all certain that all our difficulties are due to the supposedly complex character of the naturally occurring antigens, such as horse-serum, which are commonly used in the precipitation reaction. On the other hand, the antisera which we use may, almost certainly do, show wide differences in composition. If blood is taken from the fifth to the eighth day after a series of injections of an antigen, the serum may contain both antigen and antibody and may form a precipitate within a few hours of separation from the clot. Other specimens of antiserum, which presumably contain less of the antigen, do not form a spontaneous precipitate for weeks or even months. We know that a foreign serum may be detected for weeks after it has been injected into a vein. In the preparation of antiserum for the precipitation reaction relatively large amounts of foreign serum are often injected. The blood is often taken on the tenth day and seldom later than the twelfth day after the last injection. It would be remarkable if antigen were not still present in the antiserum. Antisera prepared for the precipitation reaction and very specially strong precipitating antisera very frequently show large spontaneous precipitates after they have been kept. Probably all antisera contain a certain amount of antigen, the presence of which may often be demonstrated by mixing two antisera together. As a rule antiserum collected soon after the last

injection may be expected to contain much antigen and antiserum collected after a long interval relatively less. If every antiserum contains an unknown proportion of masked antigen it is not surprising that different workers have obtained different results in their examination of the content of superfluids. Zinsser and Young (1913) consider that the existence of antigen and antibody in the same fluid without reaction may be due to the protective action of some indifferent colloid substance which is present in the mixture.

### Specificity and Practical Applications.

Kraus (1897<sup>1</sup>) insisted on the specific character of the reaction. Antisera prepared against the cholera vibrio, against typhoid and plague bacilli reacted in each case only with the cultures of the homologous micro-organism. At a meeting of the Verein für innere Medicin in 1900 Wassermann and Schütze (1900) reported a precipitation reaction for the differentiation of human, cow's and goat's milk. Shortly afterwards Uhlenhuth (1900) published a paper on the differentiation of the egg-whites of different species of birds by the precipitation method and so paved the way for the application of this reaction to forensic work in the recognition of the presence of human blood in blood-stains (Uhlenhuth, 1901<sup>1</sup>). Kowarski (1901) prepared an antiserum by injecting an extract of wheat and this antiserum gave a good precipitate when mixed with wheat extract. With rye and barley extracts the precipitation was less. With pea extract there was a slight reaction and with oats the reaction was negative. This is an early example of a group reaction. In the same year Uhlenhuth (1901<sup>2</sup>) showed that the reaction could be employed for the differentiation of the meat of different species of animals. Satisfactory results were obtained with extracts from dried and smoked flesh and the reaction has been extensively employed for the detection of fraudulent substitution of one kind of meat for another.

The study of blood relationships by the precipitation method has yielded results of great interest and value. On this subject the reader is referred to the detailed information contained in Nuttall's book (*Blood Immunity and Relationship*, Cambridge University Press, 1904). Nuttall examined 900 different sorts of blood with 30 different antisera. The very extensive investigations of Nuttall, Graham Smith and Strangeways confirmed on the basis of the serum reaction the usually accepted zoological classification and provided assistance to zoologists in disputed questions of animal relationship. With antihuman serum, Nuttall showed a close relationship between the blood of man and the higher apes but obtained less marked and easily distinguished reactions with the blood of other monkeys less closely related to man. By injecting monkeys with human blood Uhlenhuth obtained a serum which reacted with human blood but not with monkey blood and so established an ingenious method for the differentiation of nearly allied species. He also obtained antihare-serum

by injecting hare-serum into a rabbit. This method is, however, not of universal application. Horses injected with donkey-serum and sheep injected with goat-serum failed to yield precipitating antisera. If rabbits are injected with sheep- or with goat-serum antisera are obtained which yield equally good precipitates with either goat- or sheep-serum. An antisheep-serum, for example, titrated by the optimal proportions method, yields the same antigen antiserum ratio with either sheep- or goat-serum. In common with other workers we have entirely failed to separate sheep from goats by the precipitation reaction. Nicolle, Césari and Debains (1920), who use an 'optimal proportions' method, have been unable with antihorse-serum to distinguish between the serum of the horse, the donkey and the mule.

In experiments designed to differentiate between two or more substances which have antigens in common, the choice of the correct dilution of the antiserum is important. A series of dilutions of the antigen-containing substances should be prepared and set up with a series of dilutions of the antiserum. As a general rule several sets of dilutions of each of the antigen-containing fluids which have to be differentiated should be set up with various dilutions or concentrations of the antiserum. Two nearly allied antigen-containing fluids may appear indistinguishable when antiserum is used in a dilution of 1 in 5, but may be readily distinguished when the same antiserum is used in a dilution of 1 in 40. The following is a convenient exercise. Antiserum is obtained from a rabbit which has received injections with hen egg-white. The antigen-containing fluids are hen egg-white, duck egg-white and hen blood-serum. By choosing suitable antiserum dilutions it can be shown that while the antiserum reacts with all three substances, it reacts best with the homologous protein. It can also be shown that duck egg-white is nearer akin to hen egg-white than is the blood-serum of the hen.

The most notorious application of the precipitation reaction is the detection of human blood in stains on clothing and weapons. If it is intended to use the results of the precipitation reaction as evidence of the presence of human blood the greatest care must be taken. A portion of the specimen should be used for the ordinary tests for blood, the presence of corpuscles if the stain is fresh, the presence of haemoglobin pigment or one of its derivatives and the formation of blood crystals. In the case of old stains, it is convenient to treat the stain with alkali and carbon bisulphide and examine for the spectrum of reduced alkaline haematin. The material intended for the precipitation reaction should be soaked or dissolved in 0·85 per cent. sodium chloride solution. The resulting fluid must be carefully filtered. The antihuman serum should be carefully tested with known human serum immediately before its use in the actual tests with the suspected solution. The best dilution of the antiserum should be known and the proportions in which the antiserum will flocculate with known human serum should be determined. In the actual test a series of dilutions of the fluid which is to be tested and a series of dilutions of known

human serum are set up with a constant volume of the previously determined dilution of the antihuman serum. At the end of the experiment if a precipitate is formed in any of the mixtures of the antiserum with the suspected fluid the control tubes containing the antiserum without the fluid and the fluid without the antiserum must be examined with the greatest care. For forensic work the complement-fixation reaction is to be preferred to the precipitation reaction. A slightly turbid or cloudy fluid can be used in many cases without detriment and even traces of human blood give sharp-cut reactions. For details of the application of the precipitation test to forensic work the reader is referred to Uhlenhuth's numerous original articles and the work by Uhlenhuth and Weidanz : *Praktische Anleitung zur Ausfuhrung des biologischen Eiweissdifferenzierungsverfahrens, mit besondere Berücksichtigung der forensischen Blut und Fleischuntersuchung sowie der Gewinnung präcipitierender Sera*, Gustav Fischer, Jena, and to the chapters contributed to works of reference, of which the most recent is the chapter on '*Die biologische Eiweissdifferenzierung mittels der Präcipitation, mit besonderer Berücksichtigung der Technik*', by P. Uhlenhuth and S. Seiffert, in Kolle, Kraus and Uhlenhuth's *Handbuch der pathogenen Mikroorganismen*, Gustav Fischer, Jena, and Urban and Schwarzenberg, Berlin and Vienna, third edition, 1928, Vol. III, p. 365.

The precipitation reaction has been used for the detection of fraud in the manufacture of foodstuffs, for example, the adulteration of wheat-flour by potato-flour, the presence of horse- and dog-meat in sausages, the adulteration of honey (Langer, 1909) and of caviare (Kodama, 1913).

The possibility of preparing a specific antiserum against the proteins of particular organs has engaged the attention of many, but owing to the overwhelming influence of the common antigens of the blood-proteins, no clear-cut results have been obtained. The lens-proteins form an important exception. Uhlenhuth showed that an extract of lens-protein did not react with a potent antiserum prepared by the injection of the serum of an animal of the species from which the lens extract had been made. After numerous experiments he succeeded in preparing an antiserum by injecting the extract of the lens of the ox. This antiserum did not react with ox-serum, but reacted not merely with extracts of ox-lens but equally well with extracts of lenses of other mammals. As judged by the precipitation reaction there is the closest similarity between the antigens of the lenses of all tested species of mammals, birds and amphibia.

Similar relationships connect the antigenic substances contained in the milk of various mammals and in the eggs of different species of birds. That there is an organ specificity as well as a species specificity is beyond doubt.

That normal serum must be looked on as a complex antigen containing many antigenic substances is rendered probable by the fact that distinct and specific antibodies can be prepared against the various serum-proteins. Weil and Coca (1912, 1913) separated egg-albumin and egg-globulin.

Against each of these substances an antiserum was prepared. Each of these antisera reacted strongly with the substance used for immunization and less strongly with the other substance. Dale and Hartley (1916) sensitized guinea-pigs against the albumin, euglobulin and pseudo-globulin fractions of serum and demonstrated the specificity of the reaction by producing specific contractions of the excised uterus.

The alteration of protein-antigens by physical and chemical methods was initiated by Obermayer and Pick (1903, 1904, 1906). The effect of heat seems to be to lessen specificity. Zinsser and Ostenberg (1914) prepared antisera against boiled serum of ox, dog and sheep. The resulting antisera were not strictly specific.

The specificity of a protein is said to be closely associated with its aromatic radicals and may be affected by iodine, diazo and nitro-compounds. Very remarkable results have been obtained by Landsteiner and Lampl (1918). Landsteiner's results are thus summarized by Wells (1924) : 'Species specificity may be altered in many other ways than by attacking the aromatic rings of the protein : by esterification with acid alcohol, acetylating with acid or acid chlorides, or methylation by means of diazomethane. The common feature of these reactions is that the alterations occur in the salt-forming groups of the protein molecules, and they evidently do not depend solely on substitution of H-ions in the aromatic radicals. This artificial, structural specificity is marked, e.g. antiserum for methylated proteins of horse-serum may react not only with methylated proteins from most diverse animal species, but also with methylated plant-proteins. It is also possible to produce artificial protein compounds which are antigenic when injected into the species from which the protein is derived, but which engender antibodies retaining species specificity. Formaldehyde-treated rabbit-serum protein, when injected into rabbits, produces antiserum reacting with itself, but not with formaldehyde-treated proteins from other species. When the immunological relationships of these artificial compound proteins are investigated it is found that cross reactions indicate actual chemical relationship. An immune serum reacts in practice with its homologous antigen or with an antigen which has the same added group as the homologous antigen, and this particular group has the same or an adjacent location in the radical which was introduced into the protein molecule. If an immune serum reacts with some other antigen which has the same added group in a different location then the location is almost always the adjacent one. But if an antiserum reacts with an antigen which contains a different added group then the location of the added group is in most instances identical with the location of the added group in the homologous antigen. Specific behaviour is determined by the chemical structure of relatively small portions of the large antigen molecule'. According to Landsteiner, not only is the character of the added radical of importance but also its place in the molecule, that is to say the spatial relations of radicals with the molecule may determine specificity.

Precipitation reactions have been employed in the recognition of the various types of pneumococcus and streptococcus. In 1917 Dochez and Avery (1917) observed that soluble substances were formed in cultures of the pneumococcus and could be used as a reagent in the precipitation reaction. Moreover, these substances are formed in the animal body and are present in pneumococcus pus and sputum and in the urine of pneumococcus patients. Their nature is discussed in detail in Chapter VII.

### The Relation of Precipitation to Other Serum Reactions.

Whether precipitates may be formed between antigen and antibody in the living animal has often been a subject of discussion. That both antigen and antibody may be demonstrated in serum separated from blood drawn from about the fifth to the ninth day after an injection of antigen, is well known. In Opie's (1923) experiments both antigen and antibody could be demonstrated in serum obtained about the seventh day after an injection of foreign serum or egg-white. But when a purified antigen such as crystalline egg-white was injected the simultaneous presence of antigen and antibody in the serum was never demonstrated. Opie attributes the simultaneous presence of antigen and antibody in serum to the existence in normal serum of multiple antigens which on injection yield a corresponding multiplicity of antibodies.

This view was supported by Weil (1916), who stated that antigen and antibody cannot co-exist without uniting to form a precipitate, and that it was as true of the blood in a living animal as of the contents of a test-tube. On the other hand, the formation of a precipitate in the test-tube is closely connected with the proportions of antigen and antiserum present in the mixture. It is quite possible that in the body as in the test-tube antigen and antibody might co-exist without the formation of a precipitate if either antigen or antibody were present in excess. The occurrence of the phenomena of serum sickness at or about the tenth day suggests the possibility that a reaction occurs at a time when antigen and antibody are present in the body in optimal proportions. During the war signs of tetanus frequently developed in patients in spite of one or more prophylactic injections of antitoxin. Such patients were given a further injection of antitoxin and signs of serum sickness often developed four or five days later, that is to say just at the period when antibody makes its appearance after a further injection of foreign serum has been given to an immunized animal. Again, we do not know that plasma or tissue lymph is precipitable in the same way as serum is precipitable. The changes involved in the coagulation of blood and the formation of serum may be essential to the precipitability of an antiserum. If an animal is bled about seven days after the last of a series of injections of foreign protein, the antiserum is often clear when first separated from the clot, and a precipitate forms only after the lapse of some hours. Zinsser's suggestion that precipitation may be inhibited by a protective colloid is here appropriate. We know that the formation of a particulate precipitate

in a test-tube depends on a very delicate balance of antigen and antiserum in a solution of sodium chloride the strength of which is not unimportant. We do not know enough of the conditions in the living blood and living tissues to know whether precipitation is or is not likely to occur.

Although on the whole it seems improbable that the formation of a coarse precipitate can occur within the living body there is strong evidence that the capacity of an antiserum to produce passive anaphylaxis is in proportion to its titre as judged by the precipitation reaction. This relationship between these two properties of an antiserum was established by Doerr and Russ (1909), who carried out careful quantitative experiments and came to the conclusion that the amount of anaphylactic immune-body corresponds to the precipitin-content of any particular antiserum. Weil (1916) found that precipitates produced by a combination of horse-serum and of the serum of a rabbit immunized against horse-serum induce passive sensitization towards horse-serum when injected intraperitoneally into a guinea-pig. The same results hold of precipitates produced by crystalline egg-albumin and the serum of a rabbit immunized thereto. In order to be effective in inducing passive sensitization, precipitates must result from mixtures of antigen and antibody in which the proportions between the two factors do not vary outside of certain fairly wide limits. Marked relative excess or deficiency of either factor produces a precipitate which fails to sensitize passively. The sensitizing antibody is not merely adsorbed, or carried down mechanically with the precipitate. The precipitin is identical with the sensitizin.

#### The Relation between the Fixation of Complement and the Formation of a Precipitate.

The fixation of complement reaction of Bordet and Gengou has received two different explanations. According to the one view the complement is fixed by the precipitate which is formed by the union of antigen and antibody. According to the other view, precipitation and complement fixation are two entirely separate and independent phenomena. The fixation of complement is attributed to the action of an amboceptor, and some workers have gone so far as to state that a special variety—'a complement-fixing amboceptor'—exists. This amboceptor, which they call a Bordet amboceptor, is considered to be distinct from the amboceptor concerned in bacteriolysis.

Bordet and Gengou (1901<sup>2</sup>) showed that alexin was fixed by the mixture of a bacillary emulsion with its homologous antiserum. Widal and Le Sourd (1901) confirmed this result, and succeeded in demonstrating that the serum of typhoid-fever patients would bind complement in the presence of an emulsion of *B. typhosus*. Gengou (1902) extended the scope of the reaction by showing that the serum of animals which had been injected with a foreign protein had acquired the property of fixing complement if mixed with that protein. Interest in the question appears to have been revived by Moreschi (1905) who pointed out that the results which had

been attributed to the action of anticomplements were in reality due to the fixation of complement by a mixture of antiserum with its homologous protein. Neisser and Sachs (1905) applied the principles laid down by Moreschi to the production of a practical method for the identification of the blood of different animals. In subsequent papers Moreschi (1906<sup>1</sup>) and Pfeiffer and Moreschi (1906) advanced the view that the complement was taken up by the precipitate. Moreschi concluded that precipitin and precipitinogen unite in variable proportions, and form a series of precipitates, which possess a more or less strong anticomplementary action. About the same time, and independently of Moreschi, the same explanation was put forward by Gay (1905<sup>1 & 2</sup>), who came to the conclusion that the precipitate fixed the complement. In support of a criticism of the views of Neisser and Wechsberg (1901), it was shown by Gay (1905<sup>1</sup>) that the amount of interference with haemolysis was in proportion to the amount of precipitate formed, and this to the amount of antiserum present. Klein (1905) found the fixation of complement closely associated with the formation of a precipitate, and took up essentially the same position as Moreschi and Gay.

On the other hand, Neisser and Sachs (1905, 1906) found that the amount of precipitate and the capacity for binding complement stood in no direct proportions. They were able to obtain marked fixation of complement when no precipitate could be recognized. They attributed the fixation of complement to a union of the complement with the complementophile group of an amboceptor, in strict accordance with the theory of Ehrlich. The question has been critically reviewed by Muir and Martin (1906), who added the results of their experiments on the subject. Muir and Martin found that the fixation of complement was as a rule closely associated with the formation of a precipitate, but were not convinced that precipitation formed a complete explanation of complement fixation. They cited as an instance a rabbit *v.* guinea-pig-serum which produced, when mixed with its homologous antigen, a faint cloudiness, but no definite precipitate. The capacity for fixing complement, however, was possessed by this serum to a very high degree. In other experiments they found that, if the antigen was greatly diluted, no precipitate was formed, but the mixture was able to bind complement efficiently. They also observed during the process of immunizing a rabbit with human serum that the complement-binding properties of the antiserum could be demonstrated at an earlier date than the precipitating properties. Similar results were obtained by Altmann (1910), who immunized a series of rabbits with *B. paratyphosus* B. In many cases it was found that the serum of these animals developed complement-binding properties before they could be shown to have developed agglutinating properties. In the case of one rabbit only, the agglutinating property was the first to appear. After a second injection it was found that the complement-fixing property of the serum was lost during the two days immediately following, while the agglutinating property during the same period showed only a reduction.

When rabbits were injected with *B. typhosus*, it was found that the development of the two properties ran a fairly parallel course, though there was some tendency for the agglutinating property to appear earlier than the complement-fixing property. When *B. coli* was used for immunization, the power to fix complement appeared earlier in the case of nine rabbits, but in the case of two rabbits marked agglutinating power developed, but little or no power to fix complement. Altmann concluded that the discrepancy was due to differences in the individual strains of bacteria. He considered that certain strains of *B. coli* tended to induce the production of agglutinins, while others tended to produce complement-binding antibodies. Altmann regards these experiments as evidence that the agglutinins are distinct from the antibodies which are responsible for complement fixation.

Wassermann and Bruck (1905), who also considered that complement fixation was due to amboceptor action, adopted a different method with the object of supporting their view. A bacterial extract was prepared, which, when mixed with its homologous antiserum, produced a precipitate, and also a fixation of complement. This extract was set aside, and, after the lapse of some time, was found to have lost its power of producing a precipitate without any loss of its complement-binding properties. This experiment is quoted by Sachs and Altmann (1909) as conclusive proof of the separate identity of precipitins and complement-binding amboceptors.

Evidence of a similar nature was obtained by Friedberger (1906). This observer heated an antiserum, obtained by injecting a rabbit with sheep-serum, for one hour at a temperature of 67° C. This heated serum was found to have lost the property of forming a precipitate, while the property of fixing complement had been retained.

By injecting a rabbit with human serum, Friedberger obtained an antiserum so powerful that complement fixation could be obtained by the use of extraordinarily small quantities of the antigen (human serum 1 in 1,000,000,000). When these extraordinarily small amounts of antigen were used no trace of a precipitate could be detected, complement was nevertheless efficiently fixed.

The relation between the fixation of complement and the formation of a precipitate may be appreciated if an experiment is set up to show both the precipitation and complement fixation and if the same reagents and proportions are used in each reaction. In the experiment shown in Chart II, column A represents the results of the precipitin experiment. Columns B, C and D represent the results of the complement fixation experiments. Immediately after mixing turbidity appeared in tubes 2 and 3. At the expiration of ten minutes flocculi were apparent in tubes 2 and 3, and tubes 1, 4, and 5 showed uniform turbidity. After half an hour large flocculi, which had formed in tubes 2, 3 and 4, had fallen to the bottom of the tubes and left a clear supernatant fluid. Flocculi had formed in tube 1, but had separated less completely. Small particles were



visible in tube 5 and just visible in tube 6. In tubes 7 and 8 turbidity was present without visible particles. After one hour the first six tubes showed a deposit and a clear supernatant fluid. Tube 7 showed small particles suspended uniformly throughout its contents. In tubes 8, 9 and 10 a progressively diminishing opalescence was seen. After four hours all the tubes were centrifugalized and the actual amount of the deposited precipitate was read. The complement-fixing properties of the various mixtures were determined in the tubes corresponding to rows B, C and D. After one hour's incubation at 37° C. washed red corpuscles and haemolytic serum were added to all the tubes of rows B, C and D. After a further period of incubation for one hour haemolysis was read and recorded. From a study of this and similar experiments it was determined that if antigen and antibody are mixed in proportions which result in the immediate formation of a large and flocculent precipitate little or no complement is fixed. If, however, the relative proportion of antigen and antibody is such that precipitate is formed slowly complement is effectively fixed. Complement is, in fact, fixed during the earlier stages of the formation of a precipitate and the fixation of complement stands in close relationship with the rate of formation and state of division of the precipitate. That complement is indeed fixed most effectively during the earlier stages of the reaction between antigen and antibody has been shown by Dean (1911<sup>1&2</sup>) by comparing parallel sets of tubes in which complement has been present at the commencement of the reaction or been added at intervals of half or one hour after the mixture of antigen and antiserum. The relation between the proportions which favour precipitation and those which favour complement fixation vary with the strength of the precipitating serum. With only moderately strong precipitating sera, that is to say when the precipitate is formed slowly and does not appear until some minutes have elapsed after the mixture of the two reagents, the proportions most favourable to precipitation are also the proportions most favourable to complement fixation. In the case of very strong precipitating sera, such as the serum used in the experiment recorded in Chart II, the proportions which are most favourable to precipitation and in which flocculi are formed almost at once are unfavourable to the fixation of complement, and the zone of maximum fixation is found in those mixtures which contain less antigen than the amount most favourable for precipitation. The zone of complement fixation can also be altered within a series of antigen dilutions by varying the time at which the complement is added to the mixture. If complement is present when antigen and antiserum first meet, the principal zone of fixation corresponds with the optimal proportions for precipitation in the case of moderately strong sera or occurs in tubes which contain a relative antiserum excess in the case of very strong sera. Goldsworthy (1928) has shown that if an interval is allowed to elapse between the mixture of antigen and antiserum and the addition of complement, the zone of fixation corresponds with the zone of relative antigen excess. If to a series of dilutions of antigen a constant

amount of antiserum is added and the progress of precipitation is observed, precipitation with the formation of flocculi occurs in the zone of optimal proportions and shortly after in the zone of relative antiserum excess. At the end of one or two hours there is usually a zone of opalescence and turbidity in the zone of antigen excess, in the zone, in fact, which corresponds with the fixation zone when complement is added at a considerable interval after the mixture of antigen and antiserum. It has been shown in a series of experiments in which antisera of various strengths have been employed and in which the conditions of the experiment have been varied that the zone of fixation corresponds to the zone in which a very finely divided precipitate or opalescence was present during the period of fixation.

It is well known that the antiserum in a precipitation reaction must not be too highly diluted, there must, in fact, be enough of the proteins of the antiserum to constitute a visible precipitate. The visibility of a precipitate as a matter of fact depends a good deal on the conditions of the experiment and on the illumination. A precipitate may be quite invisible in 2 c.cm. of a mixture of antigen and antibody contained in an ordinary test-tube and clearly visible if the same mixture is filled into the relatively great length of a polarimeter tube. If a series of dilutions of an antiserum are prepared a dilution can be selected which when mixed with an appropriate quantity of the homologous antigen forms no precipitate but nevertheless binds complement. If complement is added to such a mixture of antigen and antiserum a precipitate appears after an interval of 6 to 24 hours. By using an appropriate mixture of antigen and antiserum the amount of the precipitate can be increased, within certain limits, by increasing the amount of guinea-pig serum (complement) present in the mixture. Similar results can be obtained if a euglobulin solution prepared from guinea-pig serum is substituted for the normal guinea-pig serum. A suitable mixture of antigen and antiserum precipitates the euglobulin of guinea-pig serum in a manner which may be compared with the precipitation of euglobulin by carbon dioxide. The particles of a precipitate probably adsorb the euglobulin of the guinea-pig serum and this adsorption of euglobulin is an essential part of the mechanism of complement fixation.

The experiments above described show that complement is fixed during the earliest stages of the reaction which takes place when antigen is mixed with antiserum. After a visible precipitate has formed little or no complement is fixed. The rate of the formation of a precipitate and consequently the size of the contained particles is influenced by the temperature in which the reaction is performed. In experiments described by Dean (1917<sup>2</sup>) it was shown that mixtures of antigen and antibody fixed more complement at 0° C. than at 37° C. In order to demonstrate effectively the difference between fixation at different temperatures it is essential that the ingredients should be brought to the required temperature before they are mixed. It was found that the maximum fixation was attained more rapidly at 37° C. than at 0° C., but the amount of complement fixed was much greater at 0° C. than at 37° C.

In all our experiments the importance of optimal proportions of antigen and antibody was found to be as important in the complement fixation as in the precipitation reaction. In the titration of antiserum by the complement-fixation method it is necessary to titrate decreasing amounts of antiserum with decreasing amounts of antigen. The importance of optimal proportions in the complement-fixation reaction has been confirmed by Parker (1923), who points out the practical point that astonishingly minute amounts of antigen or antibody may be demonstrated by complement fixation, provided that the antiserum, or, conversely, the antigen used in the experiments is employed in a series of diminishing amounts. Unless this procedure is adopted antigen or antibody may be overlooked. An example of the practical importance of optimal proportions in the precipitation reaction has been recorded recently by Goldsworthy (1928). Goldsworthy examined the serum from a case of hydatid disease. When he employed the ring test in which hydatid fluid is allowed to form a layer above undiluted serum he obtained negative results. By the application of the optimal proportions method he found that the undiluted fluid produced opalescence when mixed with the patient's serum in a dilution of 1 in 10 and definite flocculi when a serum dilution of 1 in 50 was employed. In this case, as the hydatid fluid had a low content of specific antigen it was necessary to reduce the concentration of the serum in order that optimal proportions might be attained.

#### Agglutination and Phagocytosis.

The relation between precipitation and agglutination has been often discussed. There has been in fact a general tendency to admit that these two reactions may be produced by the same antibody. Earlier theories were often coloured by the belief that the antiserum in the precipitating reaction was the active reagent and precipitated the antigen. Hence it was thought that the agglutinating serum might act by precipitating the bacteria or coagulating their contents or the fluid in their environment. The conception of a close relationship between precipitation and agglutination becomes more tenable if it is supposed that agglutination is brought about by the precipitation of the globulin of the antiserum on the surface of the bacterium. Such a skin of precipitated protein would alter the surface tension between the bacterium and the surrounding fluid and by clothing the bacterium with a relatively insoluble membrane favour agglutination. This point of view is supported by experiments of Mudd, Lucké, McCutcheon and Strumia (1929), who found that when acid-fast bacteria were sensitized with antisera the following changes in their properties could be observed : (a) increased cohesiveness, (b) decrease in surface electric potential difference, (c) decrease in wettability of bacteria by oil, and (d) increased phagocytosis.

Agglutination like precipitation depends on the presence of antigen and antisera in certain relative proportions in the mixture. In one case there

is relatively much antiserum and the proteins of the antiserum form the bulk of the deposit, in the other case the antigen is present in relatively large amount and forms the greater part of the deposit. For the agglutination of bacteria the presence of precipitable protein is necessary. This precipitable substance is usually derived from the antiserum, but may in part be derived from another serum, a normal serum, if it is added to the mixture of antigen and antibody. If to a mixture of bacillary emulsion and diluted antiserum be added a third ingredient, normal serum, the agglutination of the bacteria may be rendered more complete. This reinforcement of the proteins of the antiserum by those of a normal serum has been called conglutination. Conglutination was described independently by Muir and Browning (1906) and by Bordet and Gay (1906).

The substance present in normal serum which is able to aid the agglutination of red cells or bacteria in the presence of the homologous antiserum appears to be euglobulin (Dean, 1911<sup>3</sup>). That euglobulin may be precipitated by a mixture of antigen and antibody has been mentioned above and this euglobulin precipitation is thus a common factor in the mechanism in agglutination, complement fixation and possibly in the preparation of bacteria for phagocytosis. There are good reasons to think that all the methods by which we may demonstrate the specific changes which characterize the serum of an immune animal are closely associated with the precipitation of the globulins contained in the antiserum. By precipitation is not implied the final stage of the precipitation reaction as seen in the test-tube when large flocculi have formed a deposit, but rather the earlier stages of this aggregation of the molecules of the antiserum. When an animal is immunized with a foreign substance, an antigen, the blood-proteins undergo some modification which renders them under certain conditions precipitable in the presence of fresh quantities of the antigen. The possibility that all antigen-antibody reactions are essentially of one type has been more fully discussed by Zinsser in his book on Infection, Resistance and Immunity, third edition, p. 317, by Wells in his book on the Chemical Aspects of Immunity, p. 88, and by Dean (1917<sup>1</sup>) in the Horace Dobell Lecture. The position at the present time has been summed up by Zinsser : 'We do not wish by any means to convey the impression that we consider the "unitarian" view as absolutely and rigidly proven. We do believe, however, that the denial of such a view necessitates the assumption that the injection of a pure antigen calls forth five or six fundamentally different reactions on the part of the tissue-cells, a theory which would be justified only on the basis of incontrovertible proof. We have believed in the probable truth of the "unitarian" view for a considerable number of years, with sufficient conviction to teach it as the most likely state of affairs. And while we cannot prove it in all the ramifications of the difficult experimental problems involved, we believe it has gone far enough certainly to throw the burden of proof upon those who still cling to the separation and the conception of separate structure for agglutinins, precipitins, bacteriolysins and other antibodies'.

## REFERENCES.

ALTMANN, K., 1910, *Zbl. Bakt.*, Abt. I, Orig., **54**, 174.  
 BAYNE-JONES, S., 1917, *J. Exp. Med.*, **25**, 837.  
 BONHOFF, H. & TSUZUKI, M., 1910, *Z. ImmunForsch.*, Tl. I, Orig., **4**, 180.  
 BORDET, J., 1899, *Ann. Inst. Pasteur*, **13**, 225.  
 BORDET, J. & GAY, F. P., 1906, *Ann. Inst. Pasteur*, **20**, 467.  
 BORDET, J. & GENGOU, O., 1901<sup>1</sup>, *Ann. Inst. Pasteur*, **15**, 129; 1901<sup>2</sup>, *ibid.*, **15**, 289.  
 CALMETTE, A. & MASSOL, L., 1909, *Ann. Inst. Pasteur*, **23**, 155.  
 CHAPMAN, H. G., 1910, *Proc. Roy. Soc.*, B, **82**, 398.  
 DALE, H. H. & HARTLEY, P., 1916, *Biochem. J.*, **10**, 408.  
 DANYSZ, J., 1902, *Ann. Inst. Pasteur*, **16**, 331.  
 DEAN, H. R., 1911<sup>1</sup>, *Proc. R. Soc. Med.*, **5** (Sect. Path.), 62; 1911<sup>2</sup>, *Z. ImmunForsch.*, Tl. I, Orig., **11**, 58; 1911<sup>3</sup>, *Proc. Roy. Soc.*, B, **84**, 416; 1912, *J. Hyg., Camb.*, **12**, 259; 1917<sup>1</sup>, Horace Dobell Lecture, *Lancet*, Lond., i, 45; 1917<sup>2</sup>, *J. Path. Bact.*, **21**, 193; 1923, *Brit. Med. J.*, ii, 1033.  
 DEAN, H. R. & WEBB, R. A., 1926, *J. Path. Bact.*, **29**, 473; 1928, *ibid.*, **31**, 89.  
 DOCHEZ, A. R. & AVERY, O. T., 1917, *J. Exp. Med.*, **26**, 477.  
 DOERR, 1920, *Kolloid Z.*, **27**, 277.  
 DOERR, R. & RUSS, V. K., 1909, *Z. ImmunForsch.*, Tl. I, Orig., **3**, 181.  
 v. DUNGERN, 1903, *Zbl. Bakt.*, Abt. I, Orig., **34**, 367.  
 EISENBERG, P., 1902, *Zbl. Bakt.*, Abt. I, Orig., **31**, 773.  
 FORNET, W. & MÜLLER, M., 1910, *Z. Hyg. InfektiKr.*, **66**, 215.  
 FRIEDBERGER, E., 1906, *Deuts. med. Wschr.*, **32**, 578.  
 GAY, F. P., 1905<sup>1</sup>, *Ann. Inst. Pasteur*, **19**, 593; 1905<sup>2</sup>, *Zbl. Bakt.*, Abt. I, Orig., **39**, 603.  
 GENGOU, O., 1902, *Ann. Inst. Pasteur*, **16**, 734.  
 GEORGI, W., 1920, *Med. Klinik.*, **16**, 1053.  
 GLENNY, A. T. & OKELL, C. C., 1924, *J. Path. Bact.*, **27**, 187.  
 GLENNY, A. T. & WALLACE, U., 1925, *J. Path. Bact.*, **28**, 317.  
 GLENNY, A. T., POPE, C. G. & WADDINGTON, H., 1925, *J. Path. Bact.*, **28**, 279.  
 GOLDSWORTHY, N. E., 1928, *J. Path. Bact.*, **31**, 220, 435 and 525.  
 HITCHCOCK, C. H., 1924, *J. Exp. Med.*, **40**, 445 and 575.  
 KLEIN, A., 1905, *Wien. klin. Wschr.*, **18**, 1261.  
 KODAMA, H., 1913, *Arch. Hyg.*, Berl., **78**, 247.  
 KOWARSKI, A., 1901, *Deuts. med. Wschr.*, **27**, 442.  
 KRAUS, R., 1897<sup>1</sup>, *Wien. klin. Wschr.*, **10**, 431; 1897<sup>2</sup>, *ibid.*, **10**, 736; 1912, *Handb. d. path. Mikroorg.*, hrsg. Kolle u. Wassermann, Jena, **2**, Tl. I, 732.  
 LANDSTEINER, K. & LAMPL, H., 1918, *Biochem. Z.*, **86**, 343.  
 LANGER, J., 1909, *Arch. Hyg.*, Berl., **71**, 308.  
 MASON, V. R., 1922, *Johns Hopkins Hosp. Bull.*, **33**, 116.  
 MICHAELIS, L. & DAVIDSOHN, 1912, *Biochem. Z.*, **47**, 59.  
 MOLL, L., 1906, *Z. Exp. Path. Ther.*, **3**, 325.  
 MORESCHI, C., 1905, *Berl. klin. Wschr.*, **42**, 1181; 1906, *ibid.*, **43**, 100.  
 MORGAN, H. J., 1923, *J. Immunol.*, **8**, 449.  
 MUDD, S., LUCKÉ, B., McCUTCHEON, M. & STRUMIA, M., 1929, *J. Exp. Med.*, **49**, 779.  
 MUIR, R. & BROWNING, C. H., 1906, *J. Hyg., Camb.*, **6**, 20.  
 MUIR, R. & MARTIN, W. B. M., 1906, *J. Hyg., Camb.*, **6**, 265.  
 MYERS, W., 1900, *Lancet*, Lond., ii, 98 and *Zbl. Bakt.*, Abt. I, **28**, 237.  
 NEISSER, M. & SACHS, H., 1905, *Berl. klin. Wschr.*, **42**, 1388; 1906, *ibid.*, **43**, 67.  
 NEISSER, M. & WECHSBERG, F., 1901, *Münch. med. Wschr.*, **48**, 697.  
 NICOLLE, C., 1898, *Ann. Inst. Pasteur*, **12**, 161.  
 NICOLLE, M. & CÉSARI, E., 1920, *Ann. Inst. Pasteur*, **34**, 709.  
 NICOLLE, M., CÉSARI, E. & DEBAINS, E., 1920, *Ann. Inst. Pasteur*, **34**, 149 and 596.  
 NICOLLE, M., DEBAINS, E. & CÉSARI, E., 1919, *C.R. Acad. Sci., Paris*, **169**, 1433.  
 OBERMAYER, F. & PICK, E. P., 1903, *Wien. klin. Wschr.*, **16**, 659; 1904, *ibid.*, **17**, 265; 1906, *ibid.*, **19**, 327.  
 OPIE, E. L., 1923, *J. Immunol.*, **8**, 55.  
 PARKER, J. T., 1923, *J. Immunol.*, **8**, 223.  
 PFEIFFER, F. & MORESCHI, C., 1906, *Berl. klin. Wschr.*, **43**, 33.  
 PICK, E. P., 1902, *Beitr. chem. Physiol. Path.*, **1**, 393; 1912, *Handb. d. path. Mikroorg.*, hrsg. Kolle u. Wassermann, Jena, **1**, 685.

RAMON, G., 1922, *C.R. Soc. Biol.*, Paris, **86**, 661.  
SACHS, H. & ALTMANN, K., 1909, *Handb. d. path. Mikroorg.*, hrsg. Kolle u. Wassermann. Jena, Ergänzungsband **2**, 476.  
TCHISTOVITCH, TH., 1899, *Ann. Inst. Pasteur*, **13**, 406.  
TODD, E. W. & LANCEFIELD, R. C., 1928, *J. Exp. Med.*, **48**, 751.  
TOMCSIK, J., 1927, *Proc. Soc. Exp. Biol.*, N.Y., **24**, 813.  
TSUZUKI, M., 1910, *Z. ImmunForsch.*, Tl. I, Orig., **4**, 194.  
UHLENHUTH, P., 1900, *Deuts. med. Wschr.*, **26**, 734; 1901<sup>1</sup>, *ibid.*, **27**, 82, 260 and 499; 1901<sup>2</sup>, *ibid.*, **27**, 780.  
WASSERMANN, A. & BRUCK, C., 1905, *Med. Klinik*, **1**, 1409.  
WASSERMANN, A. & SCHÜTZE, A., 1900, *Deuts. med. Wschr.*, **26**, 178 V and 182 V.  
WEIL, R., 1916, *J. Immunol.*, **1**, 19, 35 and 47.  
WEIL, R. & COCA, A. F., 1912, *Proc. Soc. Exp. Biol. Med.*, N.Y., **9**, 102; 1913, *Z. ImmunForsch.*, Tl. I, Orig., **17**, 141.  
WELLS, H. GIDEON, 1924, *J. Immunol.*, **9**, 291.  
WELSH, D. A. & CHAPMAN, H. G., 1906<sup>1</sup>, *Proc. Roy. Soc. B*, **78**, 297; 1906<sup>2</sup>, *J. Hyg., Camb.*, **6**, 251; 1910, *Brit. Med. J.*, ii, 1510.  
WIDAL & LE SOURD, 1901, *C.R. Soc. Biol.*, Paris, **53**, 841.  
ZINSSER, H. & OSTENBERG, Z., 1914, *Proc. N.Y. Path. Soc.*, n.s., **14**, 78.  
ZINSSER, H. & YOUNG, S. W., 1913, *J. Exp. Med.*, **17**, 396.

## CHAPTER XIV. ANTIFERMENTS AND DEFENSIVE FERMENTS.

By C. H. BROWNING (UNIVERSITY OF GLASGOW).

### **Antiferments.**

THE evidence that antibodies may be developed by immunizing with ferment will be considered here ; the antifermennt action of normal sera is discussed elsewhere. Hildebrandt (1893) appears to have stated originally that immunity to emulsin could be produced in rabbits by a series of doses injected into the rectum. Animals which had been treated in this way then tolerated the subcutaneous injection of a dose which was fatal for normal individuals. Also, the instillation of a solution of emulsin into the conjunctiva of the treated rabbits did not cause an inflammatory reaction such as occurred in normal animals ; according to Bayliss (1911), however, preparations of emulsin do not necessarily produce irritative effects. Morgenroth (1899) injected sterilized solutions of rennet subcutaneously into goats. After a series of graded doses the serum of one of these animals when mixed with milk in the proportion of 1 to 50 inhibited the action of rennet to such a degree that in order to produce coagulation 200 times as much rennet had to be added as was required when, instead of antiserum, normal goats' serum was used or serum was omitted altogether. Morgenroth (1900) found also that on injecting a goat with a milk-coagulating enzyme of vegetable origin (cynarase) its serum acquired in enhanced degree the property of inhibiting the action of this ferment, whereas its inhibitory action on rennet remained practically unaltered. Similarly, antirennet serum had little effect in inhibiting the action of cynarase. In Hedin's (1912) experiments the antiserum from the rabbit *v. calf's* rennet inhibited also sheeps' rennet, but to a slighter degree, and was practically without action on the rennet of pig, horse and pike. Hedin found also that the zymogen acted well as the immunizing agent.

Thus it appears that, in certain cases at least, the serum of an animal which has been injected with an enzyme may acquire more or less specific inhibitory properties. Many attempts have been made to obtain antisera to various other enzymes, but with varying results (see Putter, 1925). In general it may be said that powerful antisera have not been obtained. Also, it seems at present to be impossible to meet the objection that the antibody develops in the serum as a reaction to some protein admixture and not to actual components of the ferment itself (Coca, 1909 ; Bayliss, 1911). The precipitum formed by the protein along with the corresponding antibody might thus be capable of fixing the ferment or a part of the ferment-complex and thereby rendering it inactive. According to Young

(1918), however, although a precipitin resulted on injecting preparations of trypsin, even when derived from the same species, there was no evidence of the development of an immune antitrypsin. Again, where a slight degree of immunity has followed injections of a solution containing ferment, it is possible that a non-specific reaction may explain the result, as in the case of trypsin, according to Kirchheim and Reinicke (1914). The attempt to confer passive immunity to ferment-containing material has proved unsuccessful ; thus Joseph and Pringsheim (1913) introduced parenterally into dogs large amounts of serum of other dogs which had been rendered immune by injections of trypsin and pancreatic tissue, but failed thereby to protect them against the injection of pancreatic tissue. Thaysen (1915) has concluded that a true antibody is not present in antirennet serum because the inhibitory action of the latter is diminished after dialysis.

#### REFERENCES.

BAYLISS, W. M., 1911, *J. Physiol.*, **43**, 455.  
COCA, A. F., 1909, *Z. ImmunForsch.*, Tl. I, Orig., **2**, 1.  
HEDIN, S. G., 1912, *Hoppe-Seyl. Z.*, **77**, 229.  
HILDEBRANDT, H., 1893, *Virchows Arch.*, **131**, 5.  
JOSEPH, H. & PRINGSHEIM, J., 1913, *Mitt. Grenzgeb. Med. Chir.*, **26**, 290.  
KIRCHHEIM, L. & REINICKE, H., 1914, *Arch. exp. Path. Pharmak.*, **77**, 412.  
MORGENROTH, J., 1899, *Zbl. Bakt.*, Abt. I, **26**, 349 ; 1900, *ibid.*, Abt. I, **27**, 721.  
PUTTER, E., 1925, *Handbuch der Biochemie des Menschen u. der Tiere*, 2nd ed.,  
**3**, 483, Jena.  
THAYSEN, A. C., 1915, *Biochem. J.*, **9**, 110.  
YOUNG, W. J., 1918, *Biochem. J.*, **12**, 499.

#### Defensive Ferments—Abderhalden's Reaction.

Weinland (1906) recorded that after a series of subcutaneous injections of cane sugar had been administered to puppies their blood-serum acquired the property of inverting saccharose. It would seem, however, that this phenomenon is by no means constant. The action of the serum was destroyed by boiling. Abderhalden (1922), proceeding on similar lines, concluded originally that the introduction into the body of various foreign substances caused the appearance in the blood plasma and serum of specific 'defensive ferments' which are capable of decomposing the particular foreign compound employed. Heating for half an hour at 56 to 58° C. destroys this property of the serum as a rule ; but the heated serum is reactivated by the addition of unheated normal serum, which alone has no action on the substance. The phenomenon was regarded as a means of depriving substances of their foreign specificity, analogous to that occurring in the alimentary tract. The category of foreign substances includes those which are ordinarily classed as antigens, in addition to other compounds of simpler constitution. It should be noted that a proteolytic action of normal serum had previously been described, but in order to demonstrate this property it was necessary to treat the serum with chloroform (Delezenne and Pozerski, 1903).

Abderhalden stated further that substances foreign to the blood, although not foreign to the individual's tissues, might lead to the development of defensive ferments. According to this supposition, the foetal elements of the placenta, which gain access to the maternal circulation, lead to development of specific ferments capable of decomposing placental tissue. This 'pregnancy reaction' has been widely investigated. In a variety of diseases also, in which degeneration of special organs occurs, it was believed that ferments were produced which could be recognized by their action on the particular parenchyma concerned. Similarly in individuals affected with malignant tumours, the blood contained ferments which decomposed the tumour proteins. Such observations opened up wide possibilities in the investigation of disease, which, however, have not been realized.

For the detection of the reaction two methods were at first employed—(1) alteration of the plane of polarization resulting when the serum acts on a solution of peptone derived from the tissue in question, and (2) the demonstration of diffusible products of the nature of amino-acids, which pass into the external fluid when the serum and the boiled substrate are left in contact in a dialyser at 37° C.; for this purpose Ruhemann's reagent 'ninhydrin' was used, which, in the presence of small amounts of  $\alpha$ -amino-acids, gives a bluish colour (see Harding with Maclean and Warneford, 1916). The difficulties which attend the making of observations will not be discussed here; the ninhydrin reaction has been extensively employed. Wollman and his co-workers (1924) have used as the indicator of proteolysis the development of indole in the treated serum after inoculation with *B. coli*. Recently the refractometer and the interferometer have been applied in such investigations, as well as the determination by chemical methods of the amino-nitrogen in the digestion mixtures. It has been noted also that in some instances where there is a positive reaction the serum becomes opaque, owing apparently to a change in the globulins, while the substrate may disintegrate.

While it is generally agreed that the diffusible bodies which react with ninhydrin are produced more readily in the presence of coagulated placenta by the serum in pregnancy than by the sera of normal non-pregnant individuals, this is not an invariable rule, nor is the property of giving a positive reaction confined to pregnant sera (see Leitch, 1914). M'Vey (1918) attempted to correlate the phenomena with other immunity reactions and made the following observations: (1) The reaction is a quantitative one; along with various proteins diffusible substances reacting to ninhydrin are produced, provided that sufficient serum is present, for example, by normal rabbit's serum acting on egg-white. (2) If the serum is heated sufficiently beforehand (60° C. for an hour), the reaction is diminished or abolished. (3) The injection of an immunizing dose of egg-white (5 c.cm. 20 per cent. solution) intravenously into rabbits causes little or no alteration in the power of their serum to give a positively reacting dialysate when tested with egg-white 7 to 10 days later. This

agrees with the findings of Frank and Rosenthal (1913). According to Bronfenbrenner (1915), however, serum of immunized rabbits which contains precipitins for egg-white or ox-serum yields a positive Abderhalden reaction along with the corresponding protein but not with the heterologous. (4) The lysis of washed ox-blood by the fresh serum of an immunized rabbit gives rise to only a slight ninhydrin reaction when the mixture is subsequently dialysed in the usual fashion ; fresh normal rabbit's serum also produces diffusible ninhydrin-reacting substances when placed in contact with ox's blood corpuscles, although practically no haemolysis occurs in this case ; immune rabbit's serum which has been previously heated (1 hour at 55° C.), when placed in contact with ox's corpuscles, does not yield a positively reacting dialysate. (5) Normal guinea-pig's fresh serum, although it contains much more complement than rabbit's serum, does not necessarily cause a positive ninhydrin reaction when it produces lysis of ox-blood sensitized with multiple doses of immune-body from the rabbit ; this confirms similar results of Frank and Rosenthal (1913). Also, McNeil and Kahn (1918) failed to find evidence of proteolysis by van Slyke's method when the serum of rabbits which had been repeatedly injected with phaseolin or edestin was allowed to act on the corresponding protein in the presence of guinea-pig's complement. Accordingly, there would not appear to be any constant correlation between the Abderhalden reaction and the usual antibody reactions.

De Waele (1914<sup>1 & 2</sup>) found that the serum may pass into the positively reacting state within a few minutes after the intravenous injection into a rabbit of a mixture of egg-white along with hydrolytic products, which causes at the same time delayed coagulation of the blood. This might be interpreted as evidence that, although not new-formed, the ferment became mobilized from some site where it existed already. De Waele concluded, however, that the substances which react with ninhydrin are derived from the serum and not from the substrate. Further, Plaut (1914), Leitch (1914) and others have shown that serum on incubation with inorganic materials, such as kaolin, barium sulphate or glass-wool, without any organic substrate, may give rise to positively reacting dialysates ; a given serum may then develop the reaction in the presence of one of these inorganic bodies, and not with another. According to Bronfenbrenner (1915) and Jobling, Eggstein and Petersen (1915) the positive reaction is due to autodigestion of the serum after its antitrypsin has been removed by contact with the substrate. Bronfenbrenner believes that a positive serum 'sensitizes' the substrate, so that when the latter is washed and placed in contact with a negative serum the latter yields a positive reaction. Jobling and his co-workers found that in certain species, e.g. guinea-pig and rabbit, protease was present in the serum, but absent from the leucocytes, whereas in man and the dog normal serum contains little or no protease, but the leucocytes are rich in this ferment.

In view of the above results, the specific nature of the reaction appears extremely doubtful, and this conclusion is supported by the work of

van Slyke, Vinograd-Villchur and Losee (1915). They determined the amino-nitrogen after the mixture of serum and placental substrate had been incubated and then protein had been removed by colloidal ferric hydrate; it was found that when digestion occurred this procedure yielded values of amino-nitrogen many times greater than the experimental error. The individual variations of both pregnant and non-pregnant sera made the results from both overlap so completely as to render the reaction, even with quantitative technique, absolutely indecisive for either positive or negative diagnosis of pregnancy. Further evidence of non-specificity was seen in the fact that carcinoma tissue was digested to about the same extent as was placenta. Wollman and his co-workers (1924) have reached similar conclusions regarding the non-specificity of the reaction. Elsesser (1916), investigating the action of immune sera on purified vegetable proteins, concluded that they tend to react more often and more strongly with their specific substrates than with any other substrate, but that the reaction is far from being absolutely specific. Abderhalden (1922), however, has maintained that the reaction is specific.

#### REFERENCES.

ABDERHALDEN, E., 1922, *Die Abderhaldensche Reaktion*, 5th ed., Berlin (contains full references to earlier work).

BRONFENBRENNER, J., 1915, *J. Exp. Med.*, **21**, 221.

DELEZENNE, C. & POZERSKI, E., 1903, *C.R. Soc. Biol.*, Paris, **55**, 327, 690.

DE WAELE, H., 1914<sup>1</sup>, *Z. ImmunForsch.*, Tl. I, Orig., **21**, 83; 1914<sup>2</sup>, *ibid.*, Tl. I, Orig., **22**, 170.

ELSESSER, O. J., 1916, *J. Infect. Dis.*, **19**, 655.

FRANK, E. & ROSENTHAL, F., 1913, *Münch. med. Wschr.*, **60**, 1425.

HARDING, V. J. & MACLEAN, R. M., 1916, *J. Biol. Chem.*, **25**, 337.

HARDING, V. J. & WARNEFORD, F. H. S., 1916, *J. Biol. Chem.*, **25**, 319.

JOBLING, J. W., EGGSTEIN, A. A. & PETERSEN, W., 1915, *J. Exp. Med.*, **21**, 239.

LEITCH, A., 1914, *Brit. Med. J.*, ii, 161, 330.

MCNEIL, A. & KAHN, R. L., 1918, *J. Immunol.*, **8**, 295.

PLAUT, F., 1914, *Münch. med. Wschr.*, **61**, 238.

VAN SLYKE, D. D., VINOGRAD-VILLCHUR, M. & LOSEE, J. R., 1915, *J. Biol. Chem.*, **23**, 377.

WEINLAND, E., 1906, *Z. Biol.*, **47**, 279.

WOLLMAN, E., LABERNADIE, E., WOLLMAN, MME E. & OSTROWSKI, J., 1924, *Ann. Inst. Pasteur*, **38**, 115.

## CHAPTER XV. ANAPHYLAXIS AND RELATED PHENOMENA.

By W. M. SCOTT (MINISTRY OF HEALTH).

### Anaphylaxis.

#### INTRODUCTION.

IT must be admitted that the study of anaphylaxis has hitherto been rather a disappointment to bacteriologists ; the hopes that its phenomena, so easily elicited in common experimental animals, might show why infective diseases are associated with such effects as fever, tissue reactions and death, have not yet been fulfilled. But, nevertheless, anaphylaxis has plainly revealed itself as one of the expressions of an antigen-antibody reaction, and is, consequently, an important subject of study to the immunologist. It differs, however, from most of the other phenomena which result from the union of antigen and antibody in that it is demonstrable only in living tissue. In the analysis of its mechanism the methods current in experimental physiology are applicable and, in fact, it is mostly to them that we owe such beginnings of elucidation as are now available. Its study has well repaid the experimental physiologist and pathologist since anaphylactic reactions have fallen into line with other disorders, illuminating the highly complex mechanism which controls the capillary circulation, especially in the skin (Lewis, 1927). And its heuristic value is not nearly exhausted. It can still bring added insight into the nature of specificity among proteins ; it provides a tool for the study of the related phenomena of serum-sickness and idiosyncrasies in man ; and the pathologist will continue to use it in analysing the various forms of allergy which result from the invasion of the body by bacteria and other living pathogenic agents. It is true that these three phenomena are not necessarily simple anaphylactic reactions and their peculiarities force us to discuss them separately, but they all depend almost certainly on antigen-antibody combination *in vivo* and are consequently only special cases. It is impossible, in the space available, to mention more than a small fraction of the experimental work which has been lavished on anaphylaxis during the past twenty years. Doerr (1922, 1929) can be recommended as a guide to the rest.

#### DEFINITION.

Anaphylaxis is best defined in terms of the experimental facts. These are :

(1) *active sensitization*, i.e. that the parenteral administration of any one of a class of substances, the *anaphylactogens* (which are generally

non-toxic and include, especially, the soluble proteins), makes animals sensitive (after a certain lapse of time, 7 to 10 days, the *incubation period*), so that injection of the same substance into their circulating blood, the *assaulting dose*, sets up a characteristic train of symptoms, the *anaphylactic shock*; injection into the subcutaneous tissue sets up a local inflammatory reaction which varies from slight oedema to complete necrosis, and is known as the phenomenon of Arthus or *local anaphylaxis*,

(2) *passive sensitization*, i.e. that the acquired susceptibility can be conveyed by the serum of the actively sensitized animal to a normal animal in which it develops after a *latent period* of 4 to 24 hours, and

(3) *desensitization*, i.e. that the susceptibility, acquired either actively or passively, can be removed by the parenteral administration of the sensitizing substance in sufficient quantity.

It may be added that, though the train of symptoms constituting anaphylactic shock is not precisely the same in different species of animals, yet it is always the same (except in degree) in animals of the same species, no matter what the nature of the anaphylactogen may be, and regardless of its pharmacological action in normal animals.

#### SYMPTOMS AND LESIONS OF ANAPHYLAXIS.

##### *In the Guinea-pig.*

###### *Acute shock.*

First known as the Theobald Smith (1904) phenomenon, the sudden death of guinea-pigs injected with antitoxic horse-serum was fully studied by Otto (1906) and by Rosenau and Anderson (1906). They showed that previous serum treatment of the animal was an essential condition, and that, in fact, it was a case of the anaphylaxis described by Richet (1902) for actino-congestine and by Arthus (1903) for serum. The guinea-pig has ever since been the animal of choice for the experimental analysis of the condition. After the intravenous, intracardiac or intracerebral assaulting dose, measuring, say, 0·01 to 0·25 c.cm. of serum, there is a prodromal period of  $\frac{1}{2}$  to 3 minutes during which the animal often sneezes, scratches its nose vigorously, becomes restless, lies down and discharges urine and faeces; acute respiratory distress then sets in and death from asphyxia, with the chest fixed in full inspiration, follows in 2 to 8 minutes from the moment of injection; intraperitoneal or subcutaneous administration is much less certain but occasionally does produce death in this way.

*Bronchospasm.* The asphyxia is due to acute spasm of the muscular coat and a consequent valve-like closure of the smaller and medium-sized bronchi (Auer and Lewis, 1910). When the thorax is opened *post mortem*, the lungs are found distended with air which cannot escape through the constricted bronchi; they are pale in colour, emphysematous, and bulge forward over the heart. The bronchospasm can be reproduced *in vitro* with the isolated, perfused and artificially ventilated lungs of a sensitive guinea-pig by adding the appropriate anaphylactogen to the perfusion

fluid (Dale, 1912). It is, therefore, due to direct action on the muscular coat and not to a central or reflex nerve stimulus. The asphyxia is purely mechanical, since, when the spasm is relieved, either naturally, as in mild degrees of shock, or by the administration of atropine, which will sufficiently relax the less severe muscular contractions, normal respiration reappears and the animal usually recovers.

The bronchi of the guinea-pig have a peculiarly thick muscular coat and this may account for the prominence of bronchospasm in the symptoms ; but plain muscle in other situations also undergoes contraction, and strips of intestine or uterus taken from a sensitized animal, suspended in a bath of Ringer's solution and attached to a recording lever, give striking pictures of the effect of contact with the anaphylactogen by addition of minute amounts to the bath (Schultz, 1910 ; Dale, 1912). (For technique *vide* Vol. IX.) There is no evidence that the central nervous system is primarily affected in anaphylaxis, though the unexpected ease with which acute shock can be produced by intracerebral or subdural injection (Besredka, 1907) has not been fully explained.

#### *Prolonged shock.*

This form is easily induced in well-sensitized animals by subcutaneous or intraperitoneal injection of suitable doses of the anaphylactogen, e.g. 2 to 5 c.cm. of serum, and lasts from  $\frac{1}{2}$  to 3 or more hours, ending with death or recovery. In contrast to the acute shock, the rhythm and depth of respiration are hardly altered ; the animal lies down at full length as if in deep sleep ; if disturbed, it makes clumsy efforts to rise but at once falls over again.

*Fall of blood-pressure.* This state of coma or semi-coma is associated with a great fall in arterial blood-pressure and in the body temperature. The latter effect, which may produce a rectal temperature of as low as 30° C., is accompanied by a diminution in the respiratory gas exchange, i.e. less CO<sub>2</sub> is given up by the lungs and less O<sub>2</sub> absorbed (personal observations), and by a depression of metabolic activity in the tissues (Abderhalden and Wertheimer, 1922).

On the other hand, a rise of temperature is caused in the sensitized guinea-pig by minute doses of the anaphylactogen (0.000,000,005 c.cm. of serum), doses much smaller than will produce a similar rise in the normal animal (0.05 c.cm.) (Friedberger and Mita, 1911).

The mechanism of the fall in blood-pressure in the guinea-pig has not been intensively studied but is almost certainly very similar to that responsible for the same phenomenon in the dog and rabbit. *Post mortem* there may be little to note except congestion of the liver and intestine with occasional petechial haemorrhages.

#### *In the Dog.*

In the dog anaphylactic death in asphyxia does not occur, and, as in the 'prolonged shock' of the guinea-pig, respiratory disturbance is of

quite minor degree. Within  $\frac{1}{2}$  to 2 minutes after the assaulting intravenous dose, e.g. 5 to 10 c.cm. of serum, the dog becomes restless, vomits freely, discharges urine and faeces and then falls down in a state of great muscular weakness to remain comatose until either death occurs, sometimes within half an hour, or slow awakening (cf. Richet, 1902). The depth and duration of the coma correspond closely with the degree and persistence of a concomitant drop in the arterial blood-pressure ; this may fall from 120 to 40 mm. Hg and may obviously be the direct cause of the clinical symptoms (Biedl and Kraus, 1909). The mechanism of the fall is one of very great interest and cannot yet be said to have been completely explained. The most important part of it, however, is due to a reaction taking place in the liver, since the drop fails to occur if this organ is excluded from the circulation (by Eck's fistula, &c.).

*The reaction in the liver* manifests itself by a spasm of the muscular coat of the hepatic veins, which is highly developed in the carnivora. The result is obstruction to the flow of blood from the liver into the vena cava, intense congestion and rise in blood-pressure in the splanchnic area, and a consequent depletion of the systemic circulation ; the liver alone may in these circumstances contain as much as 60 per cent. of the total blood (Weil, 1917). A similar fall and subsequent rise in the arterial blood-pressure can be produced in normal dogs by clamping and releasing the hepatic veins, but the fall is less pronounced than in anaphylactic shock (Simonds and Brandes, 1927). If the hepatic vein is clamped in a sensitive dog and an assaulting dose then injected intravenously, no further fall of arterial pressure occurs, but if now the hepatic circulation is released for a few seconds repetition of the mechanical arrest is followed by a more pronounced fall than that produced by the first interference. It would seem that the sensitive liver on contact with the anaphylactogen produces a substance which not only causes the intense contraction of the hepatic veins but is responsible for the maximum dilatation of capillaries throughout the body necessary to account for the profound drop in the arterial pressure. A direct consequence of the constriction of the hepatic veins is an extraordinary rise in the rate of flow of (dilute) lymph from the thoracic duct, which may reach  $5\frac{1}{2}$  times normal.

*Post mortem*, the appearances in fatal anaphylaxis of the dog are, as might be expected, a swollen and cyanotic liver and congested splanchnic viscera with both subserous and submucous haemorrhages in the intestines.

#### *In the Rabbit.*

In the rabbit anaphylactic symptoms are much more irregular than in either the guinea-pig or the dog, and their mechanism is proportionally complex. In some animals the intravenous assaulting dose, say 2 c.cm. of serum, produces death in less than five minutes, the symptoms beginning within half a minute with convulsive movements and gasping respiration ; the heart usually continues to beat after breathing has ceased but may

sometimes cease first ; there is no bronchial constriction. In others a condition of coma develops, as in the prolonged shock of the guinea-pig and dog, and may last for half an hour or more, ending either in slow recovery or in death with convulsive movements and respiratory arrest as in the acute cases. In others the symptoms may be limited to slight panting lasting only for a few minutes (Scott, 1910-11). Neither bronchospasm nor constriction of the hepatic veins appears to occur in the rabbit to any significant degree, but, both in those dying within a few minutes and in those surviving after coma, there is a profound fall of arterial blood-pressure from 50 to 10 mm. Hg or less. There is also, as in the dog, intense congestion of the splanchnic area (with local haemorrhages in the viscera), which probably accounts for a large part of the pressure fall.

*Pulmonary reaction.* The respiratory arrest is associated with a definite increase in the pressure within the pulmonary artery (Drinker and Bronfenbrenner, 1924) which has been variously explained as due to spasm of the muscular coat of the vessel and its branches, or to obstruction to the flow of blood through the lung capillaries. The cause of such an obstruction is not definitely known, but there appears to be little doubt that in the rabbit the pulmonary circulation is the site of the most intense reaction ; the intensity varies greatly, however, in different rabbits.

Fall of temperature during coma is less pronounced than in guinea-pigs (2 to 3° C. only), but the depression of respiratory gas exchange is easily demonstrated (Scott, 1910-11). *Post mortem*, congestion of the abdominal viscera with subserous and submucous petechiae is the most obvious change. The lungs are not distended as in the guinea-pig, but, on microscopical examination, they show very great swelling of the interalveolar septa with some collapse of the alveoli.

#### *In Other Animals.*

In other animals the symptoms of anaphylaxis have been less fully investigated. Mice appear to resemble rabbits in their anaphylactic response (Ritz, 1911 ; Schiemann and Meyer, 1926), while rats are difficult to sensitize (Parker and Parker, 1924 ; Kellaway, 1930). Cats are highly sensitive to intravenous injections of soluble proteins, quite apart from anaphylactic sensitization, which makes investigation difficult, but they show little sign of the hepatic vein spasm of the dog, and the brunt of the disturbance seems to fall on the lung and systemic capillaries. Cattle and horses, also, occasionally show 'natural' sensitiveness to intravenous injection of foreign protein and are easily sensitized. An assaulting dose in them produces dyspnoea and muscular weakness ; skin eruptions may appear with haemorrhages in and from mucous membranes. Monkeys are not naturally sensitive and are not easy to sensitize, though milder degrees of shock can be obtained (Zinsser, 1920, and personal observations). They probably resemble the human being in this respect, since in man, natural sensitiveness is rare, and, in acquired susceptibility, the general shock effects are seldom severe and still more seldom fatal.

*Blood Changes.*

Common to all species are certain changes in the blood in anaphylactic shock. These include diminution or complete loss of coagulability, concentration, with increase both in the amount of total protein and in the number of red corpuscles per unit volume, pronounced leucopenia and weakening of the lytic action of the complement. Of these changes, the loss of coagulability of the blood is almost certainly due to the disturbance of the liver : the concentration of the blood is due to loss of fluid through the dilated and permeable capillaries, and the leucopenia probably to adhesion of leucocytes to the damaged capillary walls. The weakening of complement, though once considered to be a reaction of special significance, is not constant and bears no relation to the symptoms ; it scarcely occurs in the acute shock of the guinea-pig, except in passive anaphylaxis, and seems to depend on the appearance in the blood-stream of antigen-antibody compounds capable of adsorbing.

*Essential Reactions.*

Finally it must be emphasized that the differences in the symptomatology of different species of animals depend on anatomical and physiological peculiarities which produce precisely similar differences in the pharmacological action of such substances as peptone and histamine, as we shall see. There are no differences in the type of cell involved ; in all species the reactions are essentially contraction of plain muscle and dilatation of capillaries and all the symptoms can probably be traced back to these.

**LOCAL ANAPHYLAXIS.**

Local anaphylaxis was first described by Arthus (1903), who injected daily doses of horse-serum subcutaneously into rabbits. The first five or six doses were absorbed without any local reaction, but all succeeding doses produced foci of oedema with surrounding inflammation, and finally a condition was reached in which a dose of 2 c.cm. of horse-serum under the skin produced an area of definite necrosis. This, the phenomenon of Arthus, is not confined to the rabbit, but can be induced in all animals including man, though, as a rule, the reaction is milder and stops short of necrosis. Local anaphylaxis in the form of lesions resembling urticaria can be produced by intradermal injections in sensitized animals ; these are of particular interest and importance in the study of idiosyncrasies (*vide* p. 485). Histologically, the phenomenon of Arthus cannot be differentiated from other forms of inflammatory reaction ; it is significant, however, that signs of damage to the capillary endothelium with relaxation and rupture are conspicuous features.

Although 'local anaphylaxis' has come to mean the skin reaction only, there is every reason to believe that other tissues, e.g. the internal organs of the sensitized animal, would show a similar inflammatory reaction on contact with antigen.

## SENSITIZATION.

*Anaphylactogens.*

We must now consider in more detail the properties of those substances which 'sensitize' the animal and determine the extraordinary difference in its behaviour to subsequent injections just described. Of these anaphylactogens the soluble proteins, such as are met with in blood-serum, egg-albumin or milk, are most commonly used for experimental anaphylaxis. Much work has been done with more highly organized materials, such as blood corpuscles, organ emulsions, bacteria and other cells, the motive being that in bacterial and other parasitic infections an anaphylactic stage may be supposed to occur in nature, though this is probably true only in a limited sense. But the physical condition and chemical complexity of such anaphylactogens make them most unsatisfactory in the study of the anaphylactic process itself, which is our present aim. Even with the soluble proteins just mentioned, the simplicity is superficial; from each of them, serum, egg-white and milk, it is possible to separate components which behave differently in the production of anaphylaxis. Purified proteins such as the crystallized albumins of serum, egg-white and vegetable seeds (edestin, &c.) are, therefore, the most satisfactory reagents.

*Qualitative factors.* All the anaphylactogens are antigens, that is, they produce immunity reactions demonstrable by in-vitro tests, and have all the characters of antigens discussed under general immunity. An essential property is that they should be 'foreign' to the circulating blood, though they need not be foreign to the animal (e.g. lens protein). All are proteins or have a protein component, though all proteins are not necessarily good anaphylactogens, e.g. haemoglobin, which is almost, if not quite, inert in that respect, while globin is strongly anaphylactogenic (Ottensooser and Strauss, 1928). Generally speaking, globulins and the vegetable proteins are good anaphylactogens; serum-albumin is less good, while muco-proteins and plant pollens are poor. There are differences in different animal species in their response to the various proteins; dogs, for example, are not easily sensitized, if at all, to egg-white, which is an excellent anaphylactogen for the guinea-pig. Species-specificity, race-specificity, probably even individual specificity (shock on repeated blood transfusion), organ-specificity (lens protein) and chemical specificity (azo-protein, nitro-protein, &c.) can all be demonstrated more or less easily in the anaphylactic experiment. Moreover, the anaphylactogens can be weakened or destroyed in the same way as antigens in general by heat, light, chemical treatment and tryptic digestion; the weakening is expressed by the necessity for larger doses in sensitization and a longer incubation period.

The toxins of diphtheria and tetanus, though eminently antigenic and probably protein in nature, are unsuitable as anaphylactogens, and it is doubtful if true anaphylaxis can be induced with them.

*Toxin supersensitiveness.* The supersensitiveness to toxin observed by Behring (1893) in the course of immunization of animals seems to be a case of genuine increased susceptibility of specifically sensitive cells ; it is, perhaps, not dependent on an antigen-antibody reaction and its symptoms have no physiological resemblance to those of protein anaphylaxis ; they appear, for the most part, as a simple exaggeration of the primary toxicity ; their relation to bacterial allergy has not been investigated.

No non-protein substance has yet been found to act as an anaphylactogen, but the possibility of modifying protein by combining it with chemicals so that it becomes a 'chemically specific' anaphylactogen (azo-proteins, &c., Landsteiner, 1924) suggests that the drug idiosyncrasies (quinine, arsenic, &c.) will eventually be proved to be examples of protein anaphylaxis of this kind. So far the only suggestive experiments directly bearing on this are those of Klopstock and Selter (1927), who have sensitized guinea-pigs to diazotized atoxyl by previous injection with simple mixtures *in vitro* of the drug and guinea-pig serum. Protein-free lipoids have not been proved to be anaphylactogens or even to form hapten compounds. The part played by 'haptens' in the genesis of protein-sensitiveness will be discussed under bacterial anaphylaxis (p. 484).

*Quantitative factors.* The minimal amount of protein required to sensitize guinea-pigs is almost incredibly small ; Wells (1929) got definite sensitization after subcutaneous injection into small guinea-pigs of the twenty-millionth part of a gramme of crystallized egg-albumin. To produce sufficient sensitization for fatal shock larger doses are necessary ; a single subcutaneous dose of 0.01 c.cm. of horse-serum or a corresponding amount of other proteins may be taken as a practical standard for this purpose. Still larger doses, on the other hand, especially if repeated, protect the animal for weeks from the effects of an assaulting dose (*vide* the 'refractory state', p. 471). The quantitative estimation of anaphylactogenic activity involves measurement either of the minimal sensitizing dose for active anaphylaxis of the guinea-pig, or, with a constant medium-sized dose, the length of time before sensitization develops. In this way Dale and Hartley (1916) showed that the euglobulin of serum possessed the highest activity ; pseudo-globulin was intermediate between it and the albumin fraction, the incubation period being progressively longer with the latter two as compared with that after an equivalent dose of the first. Doerr and Berger (1922) showed that injection of excess of euglobulin in a mixture with albumin prevents the development of anaphylaxis to the albumin fraction, but, when albumin is in excess, even to 100 times the amount of euglobulin in the sensitizing mixture, anaphylaxis to euglobulin develops normally, giving a direct demonstration of its sensitizing prepotency. It is possible, also, to measure activity by the strength of the resulting serum in passive sensitization, but animals differ so much in the height of their antibody response to the same antigen that very large numbers would be necessary to establish differences.

In rabbits the minute amounts of protein suitable for the sensitization of guinea-pigs are usually quite ineffective. Exceptionally (in young rabbits) a single dose of 3 c.cm. or less of foreign serum intravenously may sensitize so that the same dose ten days later produces fatal shock. In general, a course of injections, such as will produce high-titre precipitin serum, is necessary to sensitize full-grown rabbits, and even such a course may fail. Dogs are more easily and regularly sensitized ; 0.5 c.cm. of horse-serum per kgm. of body-weight subcutaneously, followed the day after by the same dose intravenously (Weil, 1917), will sensitize practically all dogs to some extent and sufficiently to give fatal shock in about one in four.

*Time factors.* With the standard dose of 0.01 c.cm. of foreign serum, guinea-pigs begin to be sensitive in about eight days, the maximum sensitization being reached in about three weeks. Thereafter it declines, but it remains sufficiently high to give fatal shock (with rather large assaulting doses) for six months ; some degree of sensitiveness is probably permanent. As mentioned already, larger sensitizing doses prolong the incubation period ; with 0.1 c.cm. sensitiveness may not be demonstrable for three weeks, and the maximum not attained for nearly two months. In rabbits, ten days from the last dose should elapse ; in dogs treated as described, the maximum sensitization is reached in about three weeks, and this period may be taken as the optimum interval after the last sensitizing dose for mice and most other animals.

#### *The Anaphylactic Antibody.*

The possibility of passive transference of sensitiveness to normal animals by means of the serum of a sensitized subject, as provided in the definition, proves that sensitization is associated with the appearance of a humoral antibody. The physical and chemical characters of this do not differ in any respect from those of other humoral antibodies, antitoxins, lysins, agglutinins or precipitins. It is attached, accordingly, to the globulin fraction of the serum, resists heating to 56° C. for an hour, and can be titrated by determining the minimum dose required to sensitize guinea-pigs. In practice it is found that some guinea-pigs are more easily sensitized than others of equal weight, so that the 'minimum sensitizing dose' should be expressed rather as that which will just sensitize with certainty all guinea-pigs of the experimental series employed ; quantities just below that lie in the 'border zone' of Walzer and Grove (1925). As with active sensitization, it is not safe to measure passive sensitization by the amount of the assaulting dose necessary to produce fatal shock.

Comparative experiments (Doerr and Russ, 1909) have shown that precipitin content and sensitizing titre run closely parallel. The divergencies in titre between the two manifestations of the same serum which have been observed (Falk and Caulfield, 1923) are due to the different conditions under which the two reactions are displayed, and many authorities now believe that the two activities depend on the same substance, i.e.

that there is no special anaphylactic antibody (*vide* discussions on pp. 220, 249, 279, 449).

Passive sensitization of the guinea-pig may be homologous, in which case as much as 3 c.cm. of the sensitive guinea-pig's serum is required to reach a fatal degree ; the serum of guinea-pigs which have had large and repeated doses of foreign protein (and consequently have developed precipitin) is considerably more potent, 0·1 c.cm. being sufficient ; the donor animal is itself insensitive at this stage (*vide infra*, anti-anaphylaxis). Heterologous transfer (rabbit to guinea-pig) is possible with much smaller doses ; 0·01 c.cm. of a rabbit-serum of high precipitin content suffices to render a guinea-pig fatally sensitive.

Homologous passive transfer in the rabbit is much less certain and large doses of the sensitive rabbit's serum are required, perhaps because the rabbit is inherently less responsive to the anaphylactic reaction. Dogs require even more, from one-third to two-thirds of the total blood from a sensitive dog, to become appreciably anaphylactic (Weil, 1917).

As stated in the definition, passive sensitization has a latent period. After intravenous injection of the sensitizing serum (homologous or heterologous), the animal (guinea-pig) begins to show disturbance, on injection of the foreign protein, in about 6 to 10 hours, but maximum sensitization is not reached till the fourth or sixth day (Kellaway and Cowell, 1923). A similar latent period is observed in the dog ; in the rabbit immediate sensitization may appear to occur (Scott, 1910-11), but there is some doubt whether it is a true anaphylactic shock (*vide infra*, anaphylactoid reactions, p. 472) ; in any case a latent period of as much as 48 hours is said to be necessary before the Arthus phenomenon can be induced in rabbits passively sensitized with homologous serum.

The anaphylactic antibody disappears from the circulating blood slowly in the actively, more quickly in the passively sensitized animal. In the former case (actively sensitized guinea-pigs), the blood may be free from the antibody in nine weeks, though the animal itself remains highly sensitive for many months. In passive sensitization disappearance is more rapid (following the hyperbolic curve of Madsen) ; but homologous antibody is slower to disappear completely than heterologous, which usually, though not in all species combinations, vanishes within 4 to 6 days, behaving, in fact, like any foreign protein. The disappearance from the blood, however, long precedes the elimination from the body, since sensitiveness may persist after injection with sensitive guinea-pig's serum for 8 to 10 weeks ; with heterologous sensitization (rabbit-serum into guinea-pigs), sensitiveness vanishes more quickly and has usually completely gone within 14 days, though it is still demonstrable at a time when the whole of the animal's blood can be transferred to another guinea-pig without rendering it in the least sensitive, i.e. when the antibody has completely left the blood-stream. It is interesting that, concurrently with the disappearance of anaphylaxis due to rabbit antibody, the guinea-pig becomes actively sensitive to rabbit-protein.

The mechanism whereby the blood, and eventually the whole animal, clears itself of homologous antibody is probably part of the normal process of protein metabolism ; the persistence in the actively sensitized animal is due to new-production compensating for the destruction. The more rapid disappearance of heterologous antibody may be due to its sharing the fate of the foreign protein to which it is attached and being destroyed by a special mechanism.

This period of sensitiveness in the absence of circulating anaphylactic antibody shows that the antibody can become fixed and stored in the tissues and organs while still retaining its power of reacting with the anaphylactogen. The process can be imitated *in vitro* ; Dale (1912) showed that the uterus of a normal guinea-pig gave the anaphylactic reaction after perfusion for five hours with dilute homologous sensitive serum and subsequent washing out, though one hour of perfusion failed to sensitize it. In the rat, as Kellaway (1930) has shown, homologous passive transference is difficult and exceptional, whereas heterologous, with rabbit precipitin serum, is regular and definite. Heterologous passive transference of anaphylaxis, on the other hand, is difficult or impossible with certain species combinations ; guinea-pigs cannot be passively sensitized with the serum of fowls, pigeons not with rabbit serum ; even between mammalian species similar inaptitudes are recorded ; horse antiserum will not passively sensitize guinea-pigs, at least for antipneumococcus sensitization (Avery and Tillett, 1929). There may be some connection between these inaptitudes and the curious facts already known about complement fixation ; fowl's complement will not function with mammalian amboceptor, for example, and horse amboceptor with guinea-pig's complement (in certain combinations). But the connection cannot be direct since the anaphylactic response is not apparently diminished by saline perfusion *in vitro* which must remove all but minimal traces of complement.

#### *Inheritance of Anaphylactic Sensitiveness.*

For the most part this inheritance is a case of the passive anaphylaxis just discussed. It was established by the earliest workers on anaphylaxis (Rosenau and Anderson, Otto, Gay and Southard) that crossing sensitized female guinea-pigs with normal males produced sensitive young, but that the converse crossing did not. The sensitiveness of the offspring is demonstrable from birth, and lasts, in general, for 8 to 10 weeks, which, it will be noticed, is the normal duration of homologous passive sensitization. But active sensitization of the offspring *in utero* also seems to be possible. Ratner, Jackson and Gruehl (1927) injected guinea-pigs just before the birth of their young and found that the offspring became sensitive after an incubation period of about a month : the anaphylactogen had evidently passed through the placenta. The latter point is of some importance and deserves further investigation as heredity has been regarded by some as an essential distinguishing feature of idiosyncrasies (*vide infra*) as compared with anaphylaxis proper.

*Combining Relationships of Anaphylactogen and Anaphylactic Antibody.*

The combination of these two reagents occurs both *in vitro* and *in vivo*. Combination *in vitro* is shown by mixing the serum of a sensitive animal with the appropriate protein and injecting it into a normal guinea-pig; the serum is found to have lost its power of passively sensitizing. Combination *in vivo* is demonstrated by the fact that an animal which has been passively sensitized may have its sensitization, homologous or heterologous, abolished by an appropriate dose of the corresponding anaphylactogen. In each case the amount of anaphylactogen required for neutralization is approximately proportional to the strength of antibody in the sensitive serum. But in the production of the anaphylactic shock no such direct ratio exists; the larger the amount of appropriately fixed antibody the smaller (within limits) is the amount of anaphylactogen necessary to produce fatal shock. The conditions, in fact, are exactly like those in the precipitin reaction (q.v., Chapter XIII) in which the precipitum, the resultant of the reaction, comes almost entirely from the antiserum, and the activity of the latter is measured by its sensitiveness to the antigen, i.e. by the degree of dilution of the antigen which can still produce the reaction.

Absorption experiments also are possible. For example, an animal sensitized against a protein which has group relationships with another will give a partial reaction with the group relative but continue to be sensitive to the true antigen; or its serum may be mixed with the group relative and then passively confer pure specific sensitiveness to the chosen protein on a fresh animal. The property of an anaphylactogen which makes it react with the anaphylactic antibody is not necessarily the same as its antigenic function. With this theorem, which applies also to the precipitins and lysins, is involved the demonstration of *haptenes*; these, so far as they concern anaphylaxis, will be discussed under allergy (p. 483). For example, the soluble carbohydrates of the pneumococci are not anaphylactogenic but they produce fatal shock when injected into an animal containing antibody to the whole pneumococcus.

*Reversed Anaphylaxis.*

In view of the combining relationships above mentioned, the effect of reversing the order of administration in producing passive anaphylaxis is of special interest. How does the animal with circulating antigen respond to the injection of a corresponding antiserum? In the case of the guinea-pig so treated the result is negative; no reaction occurs, and it is obvious that it is not merely the combination of the two reagents which determines anaphylactic shock but also the relation of the antibody to the tissues; until the antibody has been fixed by the tissues, cf. the 'latent period', combination does not involve disturbance of vital activities. The injection of antiserum to guinea-pig protein, which might be regarded as an analogous case, is attended with severe and fatal

anaphylactoid symptoms, but these are more properly discussed under cytotoxic sera (*vide infra*, p. 473). In the rabbit, on the other hand, 'reversed anaphylaxis' involves definite symptoms (Scott, 1910-11) and even fatal shock (Opie and Furth, 1926); these effects may be anaphylactoid in nature (*vide p. 472*). Similar behaviour is reported for mice by Schiemann and Meyer (1926). Local reactions are obtainable in the rabbit by subcutaneous injection of an antiserum after intravenous injection of the corresponding foreign protein, though the reactions are relatively mild (Opie, 1924). Subcutaneous injection of mixtures of antigen and antiserum and even of the washed precipitate resulting from their mixture also produce mild local reactions in the rabbit (Opie, 1924).

#### ANTI-ANAPHYLAXIS.

This term is hardly definable, as it has been used to cover several phenomena of quite diverse mechanism. It simply means a condition preventing, modifying or abolishing the anaphylactic reaction which would otherwise have occurred, and has been studied almost exclusively in the guinea-pig. Among such conditions the most important are as follows:

*Specific desensitization* can be achieved by injection of the anaphylactogen. This involves the risk of shock unless (*a*) it is injected subcutaneously, when shock rarely follows; the desensitizing dose for a serum-sensitive guinea-pig is about 0.01 c.cm. of serum, taking 2 to 3 hours for its complete effect; or (*b*) it is administered *in refracta dosi*, Besredka's method (1907), when even intravenous injection is safe; intermittent or continuous intravenous injection of the highly diluted antigen has the same effect. Instead of dilution, heat may be applied to the anaphylactogen: horse-serum, for example, diluted with distilled water and heated to 80° C., was shown by Besredka to desensitize without the risk of shock: the effect is probably analogous to that of dilution.

Desensitization in these ways depends simply on neutralization of the anaphylactic antibody, as is shown by the fact that the guinea-pig so desensitized can be resensitized passively just like a normal animal; moreover, an animal sensitized against more than one foreign protein can be desensitized by injection of one of them while remaining sensitive to the other or others. This specific desensitization can be effected equally well *in vitro*, and Dale (1912) and Dale and Hartley (1916) have shown this in a series of beautiful experiments with the isolated plain muscle of guinea-pigs sensitized to one or more proteins. In the rabbit specific desensitization is possible in a similar way: larger doses are necessary and the insensitive state lasts for only five days (Scott, 1910-11).

*Protection of the reacting tissue.* But, in these double-sensitization and single-desensitization experiments just mentioned, the reaction which can be induced on application of the second antigen is often definitely weaker than it should have been or may require larger quantities of the foreign protein to evoke it. The simplest explanation put forward is that the previous reaction has caused some change in the surface layer of the cells

concerned (plain muscle or endothelium) of such a nature that they are relatively irresponsible to further antigen-antibody interaction occurring in them. In this sense it might be said that the cells are ' fatigued ' to this type of reaction, and it is possible that an analogous state may be the explanation of the inhibitory effects of narcotics on the anaphylactic shock ; ether, urethane, and (intravenous) cocaine all reduce or abolish the symptoms, and, since no central nervous mechanism is concerned, their effect must be on the local reacting tissue ; perhaps the ' fatigue ' is a lowering of surface tension of the cells concerned. Similar ' fatigue ' effects may account for the difficulty of inducing skin reactions (tuberculin or serum reactions) in the measles patient during the rash : unfavourable diet, chill and intercurrent infections may also temporarily reduce anaphylactic sensitiveness, perhaps in a similar manner. Drugs such as atropine, barium chloride and adrenaline diminish or abolish the anaphylactic effects simply in virtue of their normal pharmacological action, which works in the opposite direction on the functions involved. The antagonistic effect of intravenous injection of sodium chloride, on the other hand, has been ascribed to its action in preventing or delaying antigen-antibody combination, an action which can be demonstrated *in vitro* (with precipitin sera). But it would not be safe to accept this explanation : it is more probable that the hypertonic salt, like alkalis, lipoids, saponin, &c., which also antagonize anaphylactic shock, affects in some way the physico-chemical condition of the reacting tissue, so that it is either less affected, or less capable of response on contact with the antigen. Dale (1913) showed that hypertonic salt depresses the reaction of isolated plain muscle to histamine also. The curious effects of the water of certain natural mineral springs, said to render ineffective the anaphylactic assault, may depend on some such process.

*Non-specific desensitization* has been studied especially by Kellaway and Cowell (1922, 1923). They showed that guinea-pigs, sensitized either actively or passively, could be protected against the shock of intravenous injection of the appropriate anaphylactogen by a previous injection of 1 to 3 c.cm. of normal guinea-pig's serum (or other normal serum) ; the protective effect made its appearance in about 15 minutes, reached its maximum in about an hour, and wore off entirely in something over a day. Moreover, the plain muscle of such a guinea-pig (uterus) washed free of blood, has also lost its sensitiveness, showing that the protection is due to some change induced in the reacting cells and not to a humoral inhibition. The mechanism or nature of this change is quite obscure.

*Antisensitization*, on the other hand, has at first sight an obvious explanation ; a guinea-pig receives a subcutaneous injection of normal rabbit-serum 10 days before it is passively sensitized with the serum of a sensitized (precipitin) rabbit ; on attempting to produce anaphylactic shock in the usual way after the 24 hours latent period the guinea-pig is found to be insensitive : obviously the first injection had aroused in the guinea-pig antirabbit precipitin which threw out of solution the

rabbit-serum used for passive sensitization and thus prevented its action. It is found, however, that the first injection need not be of rabbit-serum but may be of any other normal serum, which rather invalidates this explanation, and so the phenomenon may be actually as obscure as non-specific desensitization itself.

The '*refractory state*' is the name originally applied to the insensitivity to anaphylactic shock displayed by guinea-pigs which have received large and repeated doses (2 to 5 c.cm.) of the anaphylactogen (Rosenau and Anderson, 1906) and the term should be reserved for this. Such animals may remain unresponsive to the intravenous test for weeks or months, and the condition can apparently be maintained indefinitely by repeating the injection of the foreign protein at not too long intervals. According to Wells and Osborne (1914), a similar refractory state can be achieved (in the guinea-pig) by feeding with plant proteins; the animals become at first sensitive to intravenous injection of the protein, but later are refractory and even incapable of sensitization by subcutaneous injection.

The refractory guinea-pig has abundant antibody in its serum, and its serum is accordingly pre-eminently suitable for homologous passive sensitization. Moreover, its tissues (lung, uterus, intestine), after being washed free from blood by long-continued saline transfusion, are highly sensitive and respond with maximal contraction to contact with minimal concentrations of the anaphylactogen (Dale, 1912).

It is obvious that the insensitivity of such a guinea-pig depends on circumstances totally different from those determining the absence of response in the desensitized guinea-pig. What is the explanation? It is probable that the experiments of Dale and Kellaway (1922) give the clue. These observers suspended strips of uterus from passively sensitized guinea-pigs in Ringer's solution containing rabbit antiserum rich in the antibody used to produce the passive sensitiveness: addition of the anaphylactogen to the bath then failed to induce contraction and even failed to desensitize the muscle, since, after suitable washing, it contracted in the specific way on renewed application of the foreign protein. The explanation would, therefore, be that, in the refractory animal, the assaulting dose finds in the blood abundant antibody, combines with this immediately, and is, thus, unable to make contact with the sensitive tissues. Weil (1914) made experiments *in vivo* in the same direction, injecting sensitive guinea-pigs with large doses of homologous or heterologous serum of high protein-antibody titre, and finding that a high degree of protection appeared on applying the assaulting dose. It is possible, however, that part of the explanation for the refractory state in some animals may lie in a non-specific stimulation of the immunity mechanism, producing true humoral anti-anaphylactic substances, the reticulin-M of Moldovan (1928) and his collaborators.

The refractory state is not demonstrable in the rabbit, but Manwaring *et al.* (1927<sup>1</sup>) report its production in the dog (absence of anaphylactic response 24 days after the last dose of a prolonged series of injections of

horse-serum). The experiments of Manwaring and his collaborators (1925, 1927<sup>a</sup>) indicate, however, that insensitivity of the reacting tissues themselves may be the explanation in this animal, or, at least, part of it.

#### ANAPHYLACTOID REACTIONS.

Many substances can produce symptoms resembling more or less closely those of anaphylactic shock on intravenous injection into normal animals ; a list is given by Hanzlik (1924). With some of them the resemblance is superficial and the mechanism obviously different ; such are those agents which produce intravascular clotting, either gross, as with certain tissue extracts (e.g. lung tissue emulsion) and snake venoms, or microscopic as with fuller's earth, kaolin, charcoal and other coarse suspensions which cause embolism and general blockage of the pulmonary capillaries. With other substances such as toxic foreign sera (containing lysins and agglutinins for the subject's blood-cells) or suspensions of bacteria and foreign blood corpuscles (especially in immunized animals), obstruction of the lung capillaries by aggregations of the cells may account for some of the symptoms and lesions, though it must be admitted that a true anaphylactic process may also be concerned in the latter instance. Similar blockage by the specific precipitates may be the explanation of the anaphylactoid effects of precipitating sera and the phenomenon of 'reversed anaphylaxis'. Then there is a group of substances which have a common pharmacological activity as endothelial cell poisons. These include the soluble double salts of the heavy metals (platinum, gold, silver, copper, nickel, iron and cobalt) and a few organic compounds such as emetine and 'sepsin' (Faust, 1902 ; Heubner, 1907 ; Wohlwill, 1907). Intravenous injection of these substances produces symptoms and lesions closely resembling those of anaphylactic shock, and the fact is of some importance in indicating that in anaphylaxis, too, an endothelial reaction is concerned (Scott, 1910-11).

More difficult to understand are the effects of certain organic colloids, agar, starch, pectin, &c., which act either directly on intravenous injection, or indirectly and more strongly, by contact with normal guinea-pig serum *in vitro*, so that, on intravenous injection, the serum becomes lethal to the guinea-pig with symptoms of anaphylactic shock, the anaphylatoxin of Friedberger (1909).

Finally, there are substances such as Witte's peptone, which closely imitates anaphylactic shock in its pharmacological action, and histamine or histamine compounds which in all probability actually take part in the true anaphylactic reaction (*vide infra*, pp. 475, 476). On some of the anaphylactoid reactions a little more must be said.

#### *Cellular Anaphylaxis.*

Cellular anaphylaxis has been studied chiefly with erythrocytes as the anaphylactogen. Shock effects in the rabbit on repeated intravenous injections of washed red cells are familiar to all who prepare artificial

haemolysin ('amboceptor'), as also is the fact that their intensity is proportional to the amount of haemolysin present in the animal's serum at the time of injection : homologous passive transference is possible, no latent period being necessary, but specific desensitization is difficult or impossible to produce (Friedemann, 1907). Similar behaviour is reported for the dog by Kritschewsky and Friede (1907) and for the guinea-pig by Schiff and Moore (1914). Animals which possess much natural haemolysin for particular foreign blood-cells behave in the same way without preparatory treatment. Bacterial suspensions produce equivalent effects on repeated injection, but their inherent toxicity makes the superimposed anaphylactic reaction still more difficult to assess.

Intravascular clumping no doubt plays some part in the shock of cellular anaphylaxis, perhaps especially in rabbits, but the general opinion is that a true protein shock occurs, with intravascular lysis as a condition necessarily precedent. Friedli (1925) has shown that isolated plain muscle from a guinea-pig sensitized with blood-cells reacts (in the Dale experiment) to lysed corpuscles but not to the same cells intact.

The late toxic effects of cells dissolved *in vivo* (cf. the Pfeiffer phenomenon with cholera vibrios) are essentially different from the anaphylaxis with soluble protein and their mechanism is still very obscure.

#### *Cytotoxic Sera.*

Sera containing natural or artificial ('immune') cytolysin or precipitin for the cells or protein of the subject animal are highly toxic, causing local necrosis on subcutaneous injection, prolonged shock on intraperitoneal, and death within a few seconds or minutes by the intravenous route. Most of the experiments have been made on guinea-pigs, and the sera employed have been mostly from rabbits prepared by the injection of sheep's blood (serum or cells), horse kidney, guinea-pig kidney, or, in fact, any substance containing the heterogenetic antigen of Forssman (*vide p. 315*), including certain bacterial species, e.g. *B. dysenteriae* (Shiga). Quite small doses of such sera (0.1 c.cm.) cause acute death of the guinea-pig on intravenous injection ; larger doses by other routes produce the effects noted above. (Other immune sera from rabbits have not this effect, though doses of the order of 5 c.cm. injected intravenously do show a slightly increased toxicity as compared with normal rabbit-serum.) The behaviour of the guinea-pig dying after intravenous injection of serum containing the Forssman antibody and other toxic sera resembles closely acute anaphylactic shock ; the lesions, however, are somewhat different and indicate that the bronchospasm and asphyxia are secondary to an acute haemorrhagic oedema of the lung.

The explanation generally accepted (Doerr and Pick, 1913 *et al.*) is that these toxic sera combine with and damage or destroy the tissue-cells of the guinea-pig ; in the case of the heterogenetic toxic serum this combination occurs in virtue of the possession by the guinea-pig of

heterogenetic Forssman antigen. Of these tissue-cells those of the capillary endothelium of the lung are first reached by, and must bear the brunt of, an intravenous injection ; similarly when the injection is made directly into the cerebral circulation, the symptoms are primarily cerebral, with haemorrhages in the brain tissue—the ‘carotid complex’ of Forssman (1920)—and differ distinctly from the ordinary acute shock produced by similar administration of serum in a serum-sensitive guinea-pig.

*In vitro* no effect is produced by simple contact of Forssman serum with guinea-pig’s plain muscle (Redfern, 1926), though more elaborate experiments would be necessary to exclude this action *in vivo*. It seems sufficiently plain, however, that the immediate action of the cytotoxic sera, both natural and artificial is, in essentials, the same as that of the other endothelial cell poisons (heavy metals, &c.), and that, though not itself a case of anaphylactic shock, it illustrates the part played in that condition by damage to the capillary endothelium.

#### *Anaphylatoxin or Serotoxin.*

The term originated as a deduction from the experiments of Friedemann (1909), who mixed sensitized red cells with fresh rabbit-serum, centrifuged them out before lysis occurred and found that intravenous injection of the serum into normal rabbits produced symptoms of shock ; he supposed that in the early stages of lytic action the complement of the fresh serum had liberated from the cells the anaphylactic poison. Friedberger (1909), who coined the name, got similar results on incubating specific precipitates or sensitized bacteria with fresh guinea-pig serum and injecting the supernatant serum intravenously into normal guinea-pigs. There can be no doubt now that this interpretation of these experiments was false, and that the name itself should be dropped. Serotoxin, as suggested by Wells, seems to be the most satisfactory substitute.

Normal serum acquires toxic properties on contact, not only with specific antigen-antibody combinations, such as those mentioned, but also with a great variety of other substances, mostly adsorbents, e.g. coagulated albumin, bacterial masses, starch, agar and even inorganic material such as kaolin. It is obvious that the toxic property is developed not from the substrate but from the normal serum itself. The serum must be fresh, but, once the toxic property has developed, it stands drying, heating for half an hour at 56° C., and filtration through Berkefeld candles it is resistant to moderate acidification, but is readily destroyed by alkali, and, curiously enough, by prolonged contact with adsorbents such as kaolin which on shorter contact produce it. No chemical change is detectable in the serum ; in particular there is no evidence of proteolysis or histamine formation ; the serum is unaltered macroscopically, but may or may not show microscopic turbidity. The symptoms produced by intravenous injection of serotoxin (minimum fatal dose for the guinea-pig about 1·0 c.cm.) are not absolutely identical with anaphylactic

shock ; the bronchospasm and the post-mortem emphysema are slightly less pronounced, while oedema and haemorrhages in the lungs may be more prominent. A serotoxin injection renders the animal resistant to a second dose of serotoxin, but it does not protect against an anaphylactic reaction. A conspicuous blood change is the disappearance of the platelets from the circulation owing to intense clumping ; this occurs to a much less extent in anaphylactic shock. There is no evidence of thrombosis or embolism in the lung capillaries, and, in fact, if the lungs are kept artificially ventilated by increased air pressure, the lung circulation continues and the shock passes off. There is no evidence of direct stimulation of plain muscle, and the bronchospasm is probably secondary to the damage to lung endothelium. Most of these observations are due to Dale and Kellaway (1922), who conclude that the development of serotoxin is an exaggeration of a normal change which occurs in the clotting of blood, and in which the serum becomes 'foreign' to endothelium, causing it to react as in inflammation. When fresh serum is treated with the various adsorbents, this foreign condition is intensified, perhaps by ultra-microscopic precipitation increasing the extent of 'foreign' surface. There is no likelihood that any such process is involved in anaphylactic shock (except perhaps in the rabbit), since the specific reaction can be obtained in complete absence of serum, as in Dale's experiment.

The anaphylactoid symptoms which can be induced in the rabbit by intravenous injection of precipitin following intravenous injection of the antigen, 'reversed anaphylaxis', may, however, have a serotoxic basis. Injection of the precipitate itself has less effect, so that an enhanced activity of the nascent precipitum in the circulating blood must be assumed, and this would agree with the hypothesis above-mentioned. The special sensitiveness of the pulmonary capillaries in the rabbit may be an additional factor. The absence of 'reversed anaphylaxis' in the guinea-pig may be partly due to the low content of precipitin and to the physical state of guinea-pig plasma being unfavourable to precipitation in it.

#### *Peptone Shock.*

Peptone shock was first recognized by de Waele (1907) as a close imitation of the anaphylactic reaction, and has been studied by Biedl and Kraus (1909) and many others. It is evoked, especially, by Witte's peptone (a mixture of products resulting from the peptic digestion of fibrin) : the rabbit is almost quite insensitive to it, but, in the dog, intravenous injection (about 0.2 gm. per kgm.) produces the typical symptoms and lesions (turgid liver, splanchnic haemorrhages, &c.), and, in the guinea-pig (0.2 to 0.5 gm.), the characteristic bronchospasm with death by asphyxia. Moreover, peptone shock protects for some little time, not only against renewed peptone injection but against anaphylaxis and vice versa.

The analogy suggests that in anaphylaxis the animal, having acquired new powers of digesting foreign protein as the result of the sensitizing

injection, splits off similar poisonous peptone-like bodies from the protein of the assaulting dose. But no such simple explanation is possible ; there is no evidence of rapid protein cleavage occurring on the union of antigen and antibody either *in vitro* or *in vivo*, and all the theories constructed on the basis of parenteral digestion as the function of antibody and the cause of its effects *in vivo* have failed to gain acceptance (cf. Nicolle, 1920).

No recent work appears to have been done on the toxic component of Witte's peptone described as 'vasodilatin' (Popielski, 1909), and it cannot be maintained, as Dale and Laidlaw (1919) suggested, that histamine, in quantities too small for detection chemically, is responsible for more than part of the peptone effect. It seems more probable, as Dale (1929) points out, that Witte's peptone acts simply as an endothelial cell poison (cf. the 'sepsin' of Faust, 1902) with, in addition, a special action on the liver which is superposed on and greatly magnifies the endothelial reaction. It may be, also, that peptone exerts *in vivo* an action on the plasma analogous to that of adsorbents on serum *in vitro*, i.e. a serotoxin formation as suggested by Novy, De Kruif and Novy (1917).

#### *Histamine Shock.*

Histamine is a protein derivative extractable from ergot (Barger and Dale, 1911), and from many fresh animal tissues, lung, liver and skin being specially rich (Best, Dale, Dudley and Thorpe, 1927). It is usually described chemically as  $\beta$ -iminazolylethylamine, and represents the amino-acid histidine minus CO<sub>2</sub>. Many bacteria can effect this decarboxylation of histidine (Ackermann, 1910), and histamine may thus appear naturally in putrefied protein : *B. welchii*, for example, is said to produce it in cultures containing carbohydrate (Kendall and Schmitt, 1926). The vasodilator activity of alcoholic extracts of fresh animal tissues noted by many observers is a mixture of the effects of choline and histamine, which are both present in varying proportion in such organ extracts. The story of the isolation of the two activities is told in Dale's Croonian lectures (1929). In these lectures Dale also reviews the evidence for the action of histamine as a substance present in the tissue-cells and possessing a normal function in the control of the capillary circulation ; it forms one of the most fascinating chapters in the recent history of physiology.

Histamine has a pronounced toxic action, a lethal dose for a guinea-pig being about one-tenth of a mgm. intravenously : in the Dale experiment with isolated plain muscle (guinea-pig) a dilution of histamine of 1 in 5,000,000 is sufficient to cause contraction ; (it is usually employed in physiological experiments as the phosphate, three parts of the phosphate being equivalent to one of the base histamine itself). The symptoms and lesions in fatal histamine shock are remarkably like those of anaphylaxis, and vary, as do the latter, with the different animal species (Dale and Laidlaw, 1919). Skin reactions in the guinea-pig are produced only with

very high concentration of histamine (1 in 100) : the Arthus phenomenon with protein is also difficult to induce in this animal. Rabbits show the same variability to intravenous injection as in anaphylaxis, a fatal dose in one (3 mgm.) producing no symptoms in another. In dogs, the general symptoms of anaphylaxis are closely imitated, but the liver reaction is much weaker and the blood coagulation time is not appreciably lengthened. The powerful constriction of the hepatic veins which is found in anaphylactic shock in the dog is a conspicuous feature of the action of histamine (Mautner and Pick, 1915), but it can be largely abolished by adrenaline, which has little effect on the anaphylactic reaction. Isolated plain muscle of the sensitized dog reacts to suitable doses of histamine (as does the normal), but not to the specific anaphylactogen ; the possible reasons for these differences will be discussed below. In rats, which are remarkably refractory to anaphylaxis, the lethal dose of histamine is about 1,000 times greater than in the guinea-pig, and the uterine muscle *in vitro* relaxes instead of contracting on contact with it (Longcope, 1922 ; Voegtlin and Dyer, 1924). Intradermal injection in man of a 1 in 3,000 dilution produces the ' triple response ' of Lewis (1927), i.e. the primary red reaction followed by a surrounding flare and finally by an urticarial wheal, just as in the skin reaction to pressure in subjects of dermographia, to injection of pollen, &c., in asthmatics, and to the specific protein in idiosyncrasies. Subcutaneous or intramuscular injection of a small dose (0.3 mgm.) in man produces flushing of the face, outpouring of gastric juice, a slight rise of temperature and a fall of blood-pressure. The same syndrome can be elicited by skin irritation (firm stroking or brushing of large areas of skin) in dermographics (Lewis, 1927 ; Kalk, 1929). For the shock following on burns or crushing injuries, liberation of histamine may be partly responsible (the capillary poison of Bayliss), though other toxic products, no doubt, are concerned during the resorption of necrotic tissue.

On the capillaries of the frog neither histamine nor the vasodilator substance of mammalian skin have any effect, though extracts from frog's skin behave in frogs like histamine in mammals (Grant and Jones, 1929). The active principle in the frog is evidently different, but acts also as a vasodilator substance. It is probable that such principles are universal in animal cells and may even trace back to quite lowly organisms. One may speculate as to whether the association of endothelial cells and plain muscle, as the two elements susceptible to histamine, may not also be a legacy of primitive behaviour.

What is the explanation of these remarkable similarities between the pharmacological action of histamine and the anaphylactic response ? Why should the cells sensitive to histamine in each animal species be precisely those sensitive to anaphylactic shock, and hence, on the cellular hypothesis of anaphylaxis, those engaged in antibody production or fixation ? These questions demand first the consideration of the theoretical basis of the anaphylactic reaction.

## THE CAUSE OF ANAPHYLAXIS.

The facts that all anaphylactogens are antigens and that the serum of a sensitive animal can convey the specific sensitiveness in a quantitative manner establish anaphylaxis as an antigen-antibody reaction. Experimental analysis of the symptoms has shown that they depend on stimulation, (1) of the cells of unstriped muscle, and (2) of capillary endothelium, the stimulus in the former leading to contraction, in the latter to relaxation ; this combination of contraction and relaxation is not peculiar to anaphylaxis, but is found also in the action of certain poisons. The stimulation of the plain muscle is independent of the central nervous system, as shown by *in-vitro* experiments, though participation of the local sympathetic nerve plexuses cannot be excluded. In the case of the capillary endothelium, the relaxation, inferred from the fall of blood-pressure (by exclusion of other causes), from the oedema, from the increased lymph flow and consequent concentration of the blood and from direct observation of the tissues, is also independent of a central nervous mechanism, though an associated dilatation of the smallest arterioles may well be due to a local axon reflex, as it is in the action of skin irritants (Bruce, 1910).

How does the antigen-antibody union stimulate these cells ? Is it by producing changes in the fluids bathing the cells—the humoral hypothesis—or by the union occurring in or on the cells—the cellular hypothesis ?

The facts already related, (1) that an animal may give an anaphylactic reaction when its plasma contains no anaphylactic antibody (the late stages of active and passive sensitization in the guinea-pig and dog), (2) that an animal may fail to give the reaction though its blood contains abundant anaphylactic antibody (the early stage of passive sensitization or ‘latent period’), (3) that removal of blood and lymph from the tissues (guinea-pig or dog) by perfusion with saline or normal blood leaves them still fully sensitive, and (4) that tissues (plain muscle) bathed in a mixture of combining antigen and antibody do not respond, all go to show that, in these animals at least, humoral combination cannot induce the effect : the site of the reaction must necessarily be in or on the tissue-cells. Moreover, the anaphylactoid symptoms which result from antibody combination with antigen known to be fixed, i.e. cytotoxin with the tissue cells themselves, lead to a similar conclusion.

The difficulties in applying the cellular hypothesis to the rabbit and mouse are not insuperable ; the symptoms produced in passively sensitized animals without a latent period, the phenomenon of ‘reversed anaphylaxis’ and the toxic effects of antigen-antibody mixtures in these animals may have a different causal nexus ; they are, perhaps, allied to the phenomena seen with serotoxins ; in some rabbits, at any rate, the anaphylactic shock can be produced, as in the guinea-pig, after disappearance of the antibody from the blood-stream. So the probability is that anaphylactic symptoms in the rabbit are the resultant of two processes, one or other of which may predominate in different animals and under different conditions.

The failure of a humoral explanation disposes of the numerous hypotheses setting out the various ways in which a toxin might develop in the blood in consequence of the presence in it of an antigen-antibody combination, more especially as it has always proved impossible to demonstrate any toxic property in the blood obtained from animals during shock. Such theories, however, may have a place in the explanation of some of the anaphylactoid reactions and of the behaviour of rabbits and mice. The same objections apply to biophysical explanations such as that the effects are due to precipitation, gross or ultramicroscopic, of the plasma protein and deposit on the endothelial lining of the capillaries with consequent irritation, or to simple alteration of the colloidal balance of the plasma with similar consequences.

The antigen-antibody union occurs in or on the cells. How, then, does it produce its effect? It can be conceived that the protein antigen meets, in the sensitized cell, a ferment which splits off from it (within the cell) one or more of the known poisonous products of protein decomposition (cf. Abel and Kubota, 1919). This hypothesis has attractive aspects since it fits in with such observations as the increased rate of destruction of foreign protein in the immunized animal and the endothelial activity in the disposal of foreign particles; moreover, it could form part of a generalization on the defensive mechanism of the body against the introduction of non-homologous components. But the objections to it are conclusive. As Dale (1912) pointed out very early in discussing the *in-vitro* reaction of isolated plain muscle, it is inconceivable that a ferment action in the muscle-cell could proceed with the explosive rapidity necessary to account for the instantaneous contraction. Moreover, the recent observations in bacterial anaphylaxis (*vide p. 484*) show that the material of the assaulting dose need not be a protein at all, since a carbohydrate is equally potent, provided it has, as a hapten, the property of specific precipitation.

Dale's alternative suggestion was that contact with the antigen upsets the colloidal state of the protein of the muscle-fibre, which may be interpreted as intracellular precipitation analogous to that seen *in vitro*. Doerr (1922) amends that by pointing out that union of antigen and antibody may very well occur on the surface of the cell—the membrane-hypothesis—and produce a similar physical change, presumably precipitation. Though there are still certain difficulties, e.g. the assumption that the capillary endothelium is so permeable to foreign protein that it permits immediate access to the reacting tissue-cells, yet, in general, this hypothesis fits the facts, makes an easy mental picture and does away with any need for assuming the existence of the various and difficult 'toxins' which have been postulated. But it leaves two great mysteries: why should plain muscle and endothelium be the chief storehouses of antibody, as they would seem to be if the hypothesis is correct? And why should histamine affect especially, in each animal species, precisely those cells concerned in anaphylactic shock?

*The Histamine Hypothesis.*

A long step towards solution can be made by superposing on the Doerr hypothesis just mentioned the histamine theory of control of capillary circulation elaborated by Lewis (1927) and Dale (1929). Lewis, in his intensely interesting experiments on the responses of the blood-vessels of the human skin, has brought forward most convincing arguments in favour of the hypothesis that the cause of capillary reactions in skin-irritation is a preformed substance in the living epidermal cells, the H-substance. This substance is released on tissue injury even so slight as that caused by firm stroking. He concludes, from the similarity in reaction, that the H-substance is either histamine itself or a complex of similar activity, though perhaps of lower diffusibility, and supposes that the tissue injury consequent on contact with antigen in anaphylaxis liberates this substance from all cells containing antibody. Best, Dale, Dudley and Thorpe (1927) have established beyond doubt that normal tissues contain preformed histamine in varying amounts. The tissue-cells may be supposed to discharge this preformed histamine in consequence of the injury caused by antigen-antibody union in their substance or surface layer. This will act with greatest intensity on histamine-sensitive cells in the immediate neighbourhood, but, in the case of the liver and other large organs pre-eminently sensitized, the histamine will pour into the blood-stream and produce the histamine response of endothelium and plain muscle elsewhere. Any cell containing antibody and preformed histamine will liberate the latter, but only those capable of responding to histamine will give the reactions demonstrable by experimental methods. There is, thus, no necessity to restrict antibody storage to plain muscle and endothelium.

In all sensitive animals the liver is probably the organ most profoundly affected by contact with the antigen. This is certainly so in the dog, which will not give the anaphylactic reaction on introduction of the antigen into the blood-stream except when the liver is in circulation. In the guinea-pig the liver disturbance becomes evident only when the animal can survive the bronchospasm; in the rabbit it is possible that, in true anaphylactic shock, the conditions are primarily like those in the dog, and the subsequent differences in behaviour are due to differences in the response of the lung capillaries to the histamine discharged into the hepatic veins.

To explain the reactions *in vitro*, one must suppose that the plain muscle of the guinea-pig has abundant antibody, either in the muscle-cells or in the endothelial network surrounding them or both, so that contact with antigen liberates a maximal stimulating dose, whereas, in the dog or rabbit this tissue must be supposed to be almost, or quite, free from fixed antibody or have little or no preformed histamine, since their plain muscle is indifferent to the antigen *in vitro* though sensitive to histamine.

The similarity of the effects of histamine and anaphylactic shock is, thus, explained by supposing that a large part of the latter is, in fact,

histamine poisoning itself, varying, accordingly, with the natural susceptibilities to this of the different species of animal.

The two great difficulties are thus solved ; there remain, however, several phenomena incompletely explained, such as (1) the non-toxic quality of the blood removed from an animal during anaphylactic shock ; one must suppose that histamine in the blood is fixed instantaneously by the tissues, and this is supported by the fact that the blood from the hepatic vein in the anaphylactic dog does produce the histamine effect on a normal animal, though blood from the systemic circulation does not ; (2) the exceptional position of the rat's uterus, insensitive to histamine but responding with contraction when sensitized and brought in contact with the antigen, is still more difficult to reconcile with the histamine theory (cf. Kellaway, 1930) ; one must suppose that the anaphylactic reaction here is the purely direct one due to surface precipitation phenomena and the presence of precipitin in the muscle-cells. Then there is (3) the observation that histamine can produce neither the loss of coagulability of the blood nor the capillary haemorrhages which are characteristic of anaphylaxis : one must here suppose that the antigen-antibody union produces more serious damage to the cell than is necessary to liberate histamine and more serious than histamine alone can effect.

The corollary to this theory of the genesis of anaphylactic shock is that the anaphylactic symptoms have nothing peculiar to identify them except their association with the antigen-antibody reaction. Any general injury to the body-cells, especially when evoked by intravenous injection of a soluble poison, will produce a similar syndrome, resembling that produced by histamine in its primary effects, since it is due to the discharge of histamine from the injured cells, but exceeding the histamine effect later in proportion to the total damage done to the cells and their failure to recover.

### Serum-Sickness.

#### SYMPTOMS AND INCIDENCE.

Serum-sickness may appear either (1) as the rare and sometimes fatal acute shock with fainting, dyspnoea and collapse as symptoms, occasionally accompanied by vomiting, diarrhoea, cough and rapidly developing oedema of the face, throat and lung, or (2) as a simple exanthem (urticarial, morbilliform, scarlatinal, &c.), with which may be associated fever, pain and swelling in joints and lymphatic glands and some degree of albuminuria ; in the latter form complete recovery may be said to be the invariable rule.

Acute shock follows immediately upon injection of serum, especially intravenous or intraspinal, but even subcutaneous also (Dean, 1922). The exanthematous form, on the other hand, appears 6 to 14 days after the serum administration and may recur, without further serum injections, at intervals of 2 to 5 days, so that there may be three, or rarely four,

successive eruptions. There are exceptions, however, to both these rules as to incubation ; acute shock may appear, and symptoms approximating to it are common enough, at the beginning of the exanthem, i.e. after an incubation period ; and exanthematous reactions, both local and general, may appear within a few minutes of a subcutaneous serum injection, i.e. without incubation : in rare instances the immediate local reaction may be severe and progress to necrosis like the Arthus phenomenon of the rabbit. The relative frequency of the two forms is enormously different ; the acute fatal form occurs so seldom (about 1 in 100,000) as to be a pathological rarity, while the exanthematous form, appearing after a single injection of serum, affects from 20 to 90 per cent. of the subjects, the proportion increasing with the volume of serum injected and being greater in those injected intravenously.

Injection of any foreign serum or similar substance, e.g. milk, may be followed by serum-sickness. The recurring eruptions which appear in about 10 per cent. of cases of the disease are explained by the presence in serum of three or more protein fractions, each of which has its own sensitizing incubation period (Dale and Hartley, 1916). The experiments of Hooker (1923) on sensitive human skin with the protein fractions amply confirm this. These recurrences have been practically abolished by the use of 'concentrated' antitoxin in which the pseudo-globulin fraction is present almost exclusively.

Among those who have had previous injections of serum, the incidence of serum-sickness is nearly twice as high as among those receiving their first dose : their incubation period, too, is shortened, being rarely more than four days and sometimes less than one.

#### CAUSE OF SERUM-SICKNESS.

There are certain difficulties in accepting forthwith the opinion of the first observers, von Pirquet and Schick (1905), that serum-sickness is anaphylaxis in man. The obvious anomaly is that in animals the first injection of foreign protein is not followed by symptoms but the second by immediate shock ; yet in man the first injection is commonly followed by symptoms (in about 10 days), and occasionally by immediate shock, while the symptoms developing after a second injection are relatively long delayed (1 to 4 days). As regards the human reactions to a solitary injection, the immediate shock is to be regarded as due to idiosyncrasy (*q.v. infra*) ; for the exanthematous type, the usual explanation is probably correct, namely, that the foreign protein excites antibody production but itself remains in appreciable amount in the circulating blood, and, when the antibody reaches a critical value, reacts with it as in experimental protein anaphylaxis. The absence of exanthem at a corresponding period in animals is not a serious objection.

It is less easy to account for the common absence of an immediate reaction in man on subsequent injection of the specific protein. Here the serological conditions, presence of precipitin in the blood and

introduction of the corresponding antigen, are apparently the same as in the experimental animal, yet immediate symptoms rarely appear even on intravenous injection. The explanation may be twofold : (1) it may be that, as in the monkey, anaphylactic shock is inherently difficult to produce in man under these conditions (with rare exceptions) ; and (2) it may be that the skin reaction is the natural type of human anaphylaxis and requires peculiar quantitative and localizing relationships in the antibody, i.e. not too much precipitin in the circulating blood (cf. the 'refractory' guinea-pig), but a sufficient amount as fixed antibody in the epidermal tissue-cells. Experiments on passive sensitization of human skin with serum from human candidates for serum-sickness support this, e.g. those of Cooke and Spain (1929), and, though the anomaly must be recognized as unexplained by animal experiment, there is no real justification for regarding serum-sickness as anything but a special case of the anaphylactic antigen-antibody reaction.

#### PRACTICAL CONSIDERATIONS.

The use of concentrated therapeutic sera diminishes both the number and the severity of serum reactions, partly because of the smaller mass of total protein required and partly because of its lesser complexity. But caution is always indicated in repeating serum injections, especially after an interval of more than a week from the last dose, and especially intravenously. Desensitization by preliminary small doses of the serum is possible but not easy : a considerable total quantity is necessary, and may, in sensitive subjects, itself cause severe symptoms without abolishing the sensitive state : furthermore, a delay of 24 hours appears to be necessary for maximum desensitization, and such a delay in the use of therapeutic sera may have more serious consequences than the serum-sickness itself.

When intravenous or intraspinal administration has to be done, preliminary intradermal injections of the serum and dilutions of it should be made ; large wheals rapidly spreading within a few minutes and surrounded by a red 'flare', such as usually appear in subjects of 'horse-idiosyncrasy', would contra-indicate entirely, or call for the greatest caution : desensitization in such subjects is said to be almost, if not quite, impossible. Otherwise, i.e. even in presence of some skin reaction, intravenous or intraspinal injections may be regarded as free from the risk of serious shock (Park, 1924).

#### Allergy : Its Relation to Anaphylaxis.

Though originally coined by von Pirquet to cover all forms of altered behaviour after infection or immunization, allergy has come to mean in current speech the specific inflammatory reaction excited in infected animals by products of the infecting bacterium or other parasite which to normal animals are relatively inert. The tuberculin reaction is the type and has been fully dealt with by Tytler in Vol. V, p. 228, as have

the similar reactions, those of mallein, abortin, typhoidin and trichophytin, in the sections treating the corresponding species. There remains for discussion here the relation between this allergic reaction and bacterial anaphylaxis.

In the latter, as in anaphylaxis to erythrocytes and other cells, true anaphylactic shock can be produced only with dissolved cells, though intact cells can be used to sensitize : this applies to bacteria in general, including the tubercle bacillus itself. Further, the experiments with ' specific soluble substance ' from pneumococci, pneumobacilli, streptococci, &c. (cf. Tomcsik and Kurotchkin, 1928 ; Lancefield, 1928) have shown that the ' hapten ' fraction of extracts from these bacteria, which is itself non-antigenic, produces all the anaphylactic reactions in guinea-pigs (in high dilution) when these animals are passively sensitized with rabbit antisera (though not when actively sensitized). ' Old ' tuberculin, which fails to sensitize but produces the allergic reaction in animals sensitized by injection of living or dead tubercle bacilli, has an obvious resemblance to such haptens.

But the differences between the allergic reaction and the anaphylactic are very definite and not yet reconcilable on any theory of protein anaphylaxis. For example, tuberculin injected intravenously into sensitive (tuberculous) guinea-pigs does not produce anaphylactic shock with bronchospasm but simply delayed deaths (though a feeble action on the animal's plain muscle *in vitro* can be demonstrated) ; and the intra-cutaneous reaction to tuberculin (in man) is very different both in character and in rate of development from the immediate skin reaction seen in protein sensitization. Furthermore, passive transference of tuberculin sensitiveness is, if not impossible, at least difficult and rarely successful, in contrast to the ease and certainty with which protein anaphylaxis can be so transferred.

Many authors, especially Zinsser (1923), maintain, in consequence, that allergy has a different mechanism from anaphylaxis and does not depend on antigen combination with antibodies of the usual type. Zinsser (1923) would ascribe tuberculin sensitiveness to peculiar substances, developed only in the specifically infected and inflamed tissue, but distributed by the blood-stream, and, by cumulative storage, sensitizing passively all the other tissue-cells. Experiments like those of Dienes (1929) in which a tuberculin type of sensitiveness to egg-albumin was produced by injection of egg-white into tuberculous lesions would go to support this hypothesis. But similar difficulties are met with in the explanation of idiosyncrasies as due to antigen-antibody reactions, and there, as will be seen, the solution probably lies, not in postulating antibodies of different character from the precipitin type, but rather in investigating the peculiarities in the storage capacity of different tissues for the latter as ' sessile receptors ', and the corresponding anomalies in the disturbance induced on contact with the specific substance. So it will be well, for the present, to suspend judgment on the mechanism of

the bacterial allergies and to collect new evidence, bearing in mind that the hypothesis of an antigen-antibody reaction as the basis is not lightly to be abandoned.

### **Idiosyncrasies.**

Clinical and experimental study of the idiosyncrasies and, especially, of asthma, hay-fever and skin eruptions as manifestations of specific protein sensitiveness, has been so active in recent years that no adequate account of it can be given in the space available. Cranston Low (1924) and Deerr (1926), as well as the numerous publications of Coca, Cooke and their collaborators in the *Journal of Immunology* from 1919 onwards, should be consulted for further information and for the details of clinical tests. As in the case of allergy, the points discussed here are those bearing on the relation of idiosyncrasy to experimental anaphylaxis.

#### **DEFINITION, INCIDENCE, HEREDITY.**

Idiosyncrasy may be defined as abnormal sensitiveness with consequent characteristic reactions to the application, ingestion or inhalation of the exciting substance ; these substances may be of very different nature in different idiosyncrasies but the symptoms induced have a general similarity. The sensitiveness may arise apparently spontaneously under ordinary conditions of existence, or its development may be associated with special exposure to the exciting agent ; naturally occurring idiosyncrasy has been studied almost exclusively in man, and little is known as to its incidence in other animals. Its incidence in man is hard to estimate ; according to Cooke and van der Veer (1916), 7 per cent. of Americans are subjects of idiosyncrasy ; half of them express it by attacks of hay-fever and asthma and are classed by Coca as suffering from 'atopy' ( $\alpha\tau\delta\pi\iota\alpha$  = a strange disease).

There is evidence that the predisposition to become subjects of idiosyncrasy may be hereditary, since hay-fever, asthma and other manifestations run in families, but there is no sure ground as to the genetic mechanism (dominant or recessive genes), and, as in tuberculosis, other factors may be of importance in determining familial incidence. There is this, however, that, in families subject to idiosyncrasies, the different members are often sensitive to different substances, showing that it is not an abnormal exposure which determines sensitization but some constitutional influence. The possibility that tuberculosis may itself be one of the familial factors predisposing to the development of idiosyncrasy has not received sufficient attention.

It is obviously impossible to be sure that an idiosyncrasy is truly spontaneous in its origin, i.e. that it is manifested on the first contact with the exciting substance, and the fact that special exposure enormously increases the incidence of sensitiveness, as in flour-millers, furriers, drug-handlers, &c., indicates that a sensitizing contact, either with the substance itself or with some chemical ally, is probably an invariable precursor.

Another fact pointing in the same direction is the common development of idiosyncrasy in late adolescence and during maturity. Further, experimental production of sensitiveness (both local and general) in man by single or repeated contacts is easily effected, for example with the primulas and other dermatitis-producing plants (*vide* Cranston Low), though this may be a special case, as maintained by Coca.

The assumption of previous contact as the deciding factor in idiosyncrasy, as in anaphylaxis, may be accepted as, at least, highly probable, though there remain many obscurities still to explain. Why, for example, if the essential factor is a general predisposition to become sensitive, should the idiosyncrasy often be developed against only one substance, and one to which no special exposure can be alleged, e.g. strawberries, fish, milk, eggs, &c.?

The symptoms in idiosyncrasy, though extremely variable in different persons, e.g. bronchospasm, nasoconjunctival irritation, gastro-enteritis, skin lesions of great variety (including urticaria, erythema, angio-neurotic oedema) and general shock and collapse (especially after alimentary absorption), all find counterparts in the anaphylactic reaction in different animals and are all indications of disturbance in plain muscle, in capillary endothelium or in both.

#### PASSIVE TRANSFERENCE.

The successful passive transference of the specific sensitiveness in idiosyncrasy is of special importance in the comparison with anaphylaxis. A convenient method, first firmly established by the work of Prausnitz and Küstner (1921), consists in the passive sensitization of a small spot of the skin of a normal person by intradermal injection of the serum (0.1 c.cm.) of the subject of idiosyncrasy. After a latent period of 2 to 48 hours, the sensitive spot, on the injection of the exciting substance into it, gives the ' triple response ' of Lewis, i.e. a local histamine reaction. A few normal people (say 10 per cent.) appear to be incapable of sensitization thus, but a more important fact is that in many of the idiosyncrasies, passive transference to lower animals, whether by local skin injections or by the ordinary technique of passive anaphylaxis, is impossible, even in the case of the apes, though transference from man to man succeeds. In other cases even homologous transfer fails. Cooke and Spain (1929) have shown that the serum of a horse-asthmatic fails to sensitize guinea-pigs, though it will sensitize human skin, whereas the serum of a case of serum-sickness will sensitize both.

A special term, the ' reagin ', has, therefore, been adopted for the hypothetical antibody responsible for passive transference of idiosyncrasy, to distinguish it from the anaphylactic antibody (sensibilisin). These reagins behave, however, in all respects except heterologous sensitization, like the latter ; there is a latent period during which fixation of the reagin to the tissue-cells takes place and the sensitization passes off slowly, as in homologous passive anaphylaxis, in 4 to 6 weeks ; the serum

retains its property for some time at 56° C., can be stored for months at low temperatures and can neutralize the exciting substance on contact in suitable proportion *in vitro*. The reagin is, therefore, in all probability an antibody similar in nature and origin to the immune-body produced artificially.

Homologous passive transference to the skin by the method of Prausnitz and Küstner is invariably successful in those cases of idiosyncrasy in which the skin of the patient himself gives a specific reaction on intradermal test with the exciting substance, and this no matter what the nature of the patient's malady, i.e. in hay-fever and asthmatic subjects as well as in those in whom the idiosyncrasy is shown by skin eruptions or gastro-enteritis ; the primula-toxicodendron group forms an exception. Those persons who fail to give skin reactions to the substance which is certainly the cause of their symptoms have no reagin demonstrable in the passive test : it must be presumed that they have sensitized tissue, e.g. nasal mucous membrane in hay-fever, but no circulating antibody, just as the guinea-pig in the late stages of active sensitization has sensitive plain muscle but no power of passive transference.

Reagins can be demonstrated (by homologous passive sensitization, and in some cases also by guinea-pig experiment) not only against protein substances (pollen, milk, eggs, meat, fish, epithelium or hair from horses, rabbits, &c., and feathers from different birds) but against simpler chemical substances such as iodine, arsenic, mercury and bismuth compounds, quinine and its derivatives, aspirin, &c. In the case of the drug group, the antigenic mechanism is unknown ; it may be that the drugs form combinations having antigenic activity with the subject's own protein, as in the experiments of Landsteiner with haptens.

Desensitization by contact with the exciting substance, though it may be presumed to occur, since an attack protects for some time from the effects of renewed contact, is not demonstrable by the passive test : in fact, as in the 'refractory' guinea-pig, the serum during this state of temporary immunity may be more powerful than before as a passive sensitizer.

The explanation would be that the attack, i.e. the introduction of 'antigen', disturbs the balance between circulating and fixed reagin, increasing the former (protective) at the expense of the latter (sensitizing) and so favouring passive transfer, while making the patient himself insensitive.

#### MECHANISM.

All the facts set forth above, and, especially, the existence and behaviour of the reagin, support the assumption that idiosyncrasy, no matter what form its clinical manifestation may take, is simply human anaphylaxis. The anomalies still unexplained, such as the peculiar localization of reacting tissue and the absence of reagin in certain instances of idiosyncrasy, are not incompatible with the facts of experimental

anaphylaxis and may well depend on a special affinity for the antibody on the part of certain tissues and a consequent difficulty in maintaining its existence in the body fluids. Much is still to be learned, however, as to the manner in which such special affinities are created.

## REFERENCES.

ABDERHALDEN, E. & WERTHEIMER, E., 1922, *Pfluegers Arch.*, **195**, 487.  
 ABEL, J. J. & KUBOTA, S., 1919, *J. Pharmacol.*, **18**, 243.  
 ACKERMANN, 1910, *Hoppe-Seyl. Z.*, **85**, 504.  
 ARTHUS, M., 1903, *C.R. Soc. Biol.*, Paris, **55**, 817.  
 AUER, J. & LEWIS, P., 1910, *J. Exp. Med.*, **12**, 151.  
 AVERY, O. T. & TILLETT, W. S., 1929, *J. Exp. Med.*, **49**, 251.  
 BARGER, G. & DALE, H. H., 1911, *J. Physiol.*, **41**, 499.  
 BEHRING, E. VON, 1893, *Deuts. Med. Wschr.*, 678, 1253.  
 BESREDKA, A., 1907, *Ann. Inst. Pasteur*, **21**, 950.  
 BEST, C. H., DALE, H. H., DUDLEY, H. W. & THORPE, W. V., 1927, *J. Physiol.*, **62**, 397.  
 BIEDL, A. & KRAUS, R., 1909, *Wien. klin. Wschr.*, **22**, 363.  
 BRUCE, A. N., 1910, *Archiv. f. exp. Path. Pharmak.*, **63**, 424.  
 COOKE, R. A. & SPAIN, W. C., 1929, *J. Immunol.*, **17**, 295.  
 COOKE, R. A. & VAN DER VEER, A., 1916, *J. Immunol.*, **1**, 201.  
 DALE, H. H., 1912, *J. Pharmacol.*, **4**, 167; 1913, *ibid.*, **4**, 517; 1929, *Lancet*, Lond., i, 1179, 1233, 1285.  
 DALE, H. H. & HARTLEY, P., 1916, *Biochem. J.*, **10**, 408.  
 DALE, H. H. & KELLAWAY, C. H., 1921, *Proc. Physiol. Soc., J. Physiol.*, **54**, cxliii; 1922, *Philos. Trans.*, **211**, 273.  
 DALE, H. H. & LAIDLAW, P. P., 1919, *J. Physiol.*, **52**, 355.  
 DEAN, H. R., 1922, *J. Path. Bact.*, **25**, 305.  
 DIENES, L., 1929, *J. Immunol.*, **17**, 531.  
 DOERR, R., 1922, *Ergebn. Hyg. Bakt.*, **5**, 71; 1926, *Handb. d. Inner. Medizin*, hrsg. Bergmann & Staehelin, **4**, 448; 1929, *Handb. d. path. Mikroorg.*, hrsg. Kolle, Kraus u. Uhlenhuth, Jena, **1**, 759.  
 DOERR, R. & BERGER, W., 1922, *Z. Hyg. InfektKr.*, **96**, 190, 258.  
 DOERR, R. & PICK, R., 1913, *Z. ImmunForsch.*, Tl. I, Orig., **19**, 251.  
 DOERR, R. & RUSS, V. K., 1909, *Z. ImmunForsch.*, Tl. I, Orig., **8**, 181.  
 DRINKER, C. K. & BRONFENBRENNER, J., 1924, *J. Immunol.*, **9**, 397.  
 FALK, I. S. & CAULFIELD, M. F., 1923, *J. Immunol.*, **8**, 239.  
 FAUST, E. S., 1902, *Arch. exp. Path. Pharmak.*, **51**, 248.  
 FORSSMAN, J., 1920, *Biochem. Z.*, **110**, 164.  
 FRIEDBERGER, E., 1909, *Z. ImmunForsch.*, Tl. I, Orig., **4**, 636.  
 FRIEDBERGER, E. & MITA, S., 1911, *Z. ImmunForsch.*, Tl. I, Orig., **10**, 216.  
 FRIEDEMANN, U., 1907, *Münch. med. Wschr.*, **54**, 2414; 1909, *Z. ImmunForsch.*, Tl. I, Orig., **2**, 591.  
 FRIEDLI, 1925, *Z. Hyg. InfektKr.*, **104**, 233.  
 GRANT, R. T. & JONES, T. DUCKETT, 1929, *Heart*, **14**, 337.  
 HANZLIK, P. J., 1924, *J. Amer. Med. Ass.*, **82**, 2001.  
 HEUBNER, W., 1907, *Arch. exp. Path. Pharmak.*, **56**, 370.  
 HOOKER, S. B., 1923, *J. Immunol.*, **8**, 469.  
 KALK, H., 1929, *Klin. Wschr.*, **8**, 64.  
 KELLAWAY, C. H., 1930, *Brit. J. Exp. Path.*, **11**, 72.  
 KELLAWAY, C. H. & COWELL, J. S., 1922, *Brit. J. Exp. Path.*, **3**, 268; 1923, *ibid.*, **4**, 255.  
 KENDALL, A. I. & SCHMITT, F. O., 1926, *J. Infect. Dis.*, **39**, 250.  
 KLOPSTOCK, A. & SELTER, G. E., 1927, *Zbl. Bakt.*, Abt. I, Orig., **104**, 140.\*  
 KRITSCHEWSKY, J. L. & FRIEDE, K. A., 1925, *Zbl. Bakt.*, Abt. I, Orig., **96**, 56, 68.  
 LANCEFIELD, R. C., 1928, *J. Exp. Med.*, **47**, 843, 857.  
 LANDSTEINER, K., 1924, *J. Exp. Med.*, **39**, 631.  
 LEWIS, T., 1927, *The Blood-Vessels of the Human Skin and their Responses*, London.  
 LONGCOPE, W. T., 1922, *J. Exp. Med.*, **36**, 627.

LOW, R. CRANSTON, 1924, *Anaphylaxis and Sensitisation*, Edinburgh, W. Green & Son, Ltd.

MANWARING, W. H., HOSEPIAN, V. M., O'NEILL, F. & MOY, H. B., 1925, *J. Immunol.*, **10**, 575.

MANWARING, W. H., SHUMAKER, P. W., WRIGHT, R. W., REEVES, D. L. & MOY, H. B., 1927<sup>1</sup>, *J. Immunol.*, **13**, 59; 1927<sup>2</sup>, *ibid.*, **13**, 63.

MAUTNER, H. & PICK, E. P., 1915, *Münch. med. Wschr.*, **62**, 1141.

MOLDOVAN, J., 1928, *C.R. Soc. Biol.*, Paris, **98**, 1617.

NICOLLE, M., 1920, *J. State Med.*, **28**, 293.

NOVY, F. G., DE KRUIF, P. H. & NOVY, R. L., 1917, *J. Infect. Dis.*, **20**, 499, 776.

OPIE, E. L., 1924, *J. Immunol.*, **9**, 231, 247, 255, 259.

OPIE, E. L. & FURTH, J., 1926, *J. Exp. Med.*, **43**, 469.

OTTENSOOSER, F. & STRAUSS, E., 1928, *Biochem. Z.*, **193**, 426.

OTTO, R., 1906, *Leuhold Gedenkschrift*, Berlin.

PARK, W. H., 1924, *J. Immunol.*, **9**, 17.

PARKER, J. T. & PARKER, F., JR., 1924, *J. Med. Res.*, **44**, 263.

PIRQUET, C. VON & SCHICK, B., 1905, *Die Serumkrankheit*, Leipzig.

POPIELSKI, L., 1909, *Pfluegers Arch.*, **126**, 483.

PRAUSNITZ, C. & KÜSTNER, H., 1921, *Zbl. Bakt.*, Abt. I, Orig., **86**, 160.

RATNER, B., JACKSON, H. C. & GRUEHL, H. L., 1927, *J. Immunol.*, **14**, 249.

REDFERN, W. W., 1926, *Amer. J. Hyg.*, **6**, 276.

RICHET, C., 1902, *C.R. Soc. Biol.*, Paris, **54**, 837; 1923, *L'Anaphylaxie*, Paris.

RITZ, H., 1911, *Z. ImmunForsch.*, Tl. I, Orig., **9**, 321.

ROSENAU, M. J. & ANDERSON, J. F., 1906, *Bull. U.S. Hyg. Lab.*, No. **29**.

SCHIEMANN, O. & MEYER, H., 1926, *Z. Hyg. InfektKr.*, **106**, 607.

SCHIFF, F. & MOORE, H. F., 1914, *Z. ImmunForsch.*, Tl. I, Orig., **22**, 618.

SCHULTZ, W. H., 1910, *J. Pharmacol.*, **2**, 221.

SCOTT, W. M., 1910-11, *J. Path. Bact.*, **15**, 31.

SIMONDS, J. P. & BRANDES, W. W., 1925, *Amer. J. Physiol.*, **72**, 320; 1927, *J. Immunol.*, **13**, 1.

SMITH, TH., 1904, *J. Med. Res.*, **13**, 341.

TOMCSIK, J. & KUROTCHKIN, T. J., 1928, *J. Exp. Med.*, **47**, 379.

VOEGTLIN, C. & DYER, H. A., 1924, *J. Pharmacol.*, **24**, 101.

WAELE, H. DE, 1907, *Bull. Acad. Méd. Belg.*, **21**, 715.

WALZER, M. & GROVE, E. F., 1925, *J. Immunol.*, **10**, 483.

WEIL, R., 1914, *J. Med. Res.*, **30**, 87, 299; 1917, *J. Immunol.*, **2**, 399, 469, 525.

WELLS, H. G., 1929, *The Chemical Aspects of Immunity*, 2nd ed., New York.

WELLS, H. G. & OSBORNE, T. B., 1914, *J. Infect. Dis.*, **14**, 377.

WOHLWILL, F., 1907, *Arch. exp. Path. Pharmak.*, **58**, 403.

ZINSSER, H., 1920, *Proc. Soc. Exp. Biol.*, N.Y., **18**, 57; 1923, *Infection and Resistance*, 3rd ed., New York.

ZINSSER, H. & MUELLER, J. H., 1925, *J. Exp. Med.*, **41**, 159.

## CHAPTER XVI. THE BEARING OF COLLOIDAL CHEMISTRY UPON SOME IMMUNITY REACTIONS.

BY DOROTHY B. STEABBEN (LISTER INSTITUTE, LONDON).

### *Introduction.*

SINCE the phenomena of life are essentially dynamic, the recent development of colloidal chemistry, which is concerned not so much with structure as with changes of state, has made it possible to arrive at what is probably a truer conception of the forces to which a living organism is subject. Many of the problems of immunity have accordingly been restated as problems of protein behaviour, and are discussed in terms of colloid chemistry.

Immunological reactions, which it was at one time attempted to explain upon a purely chemical basis, show analogies with the known and comprehensible behaviour of colloidal substances—analogies which suggest that as these reactions become more accurately reproducible, they will also become more amenable to interpretation. The existing relation between colloidal chemistry and immunology consists largely in a translation of the descriptions of known phenomena into a language new to immunology, with one great advantage—that the language deals with substances and conditions which can be verified by laboratory experiment, and not with purely hypothetical substances to be apprehended only by their causes and effects. This translation is concerned chiefly with the comparison of the physical characters of normal and pathological sera ; with the determination of analogies between serum reactions and experimentally produced colloidal reactions in which all the factors are known and under control ; and with attempts to show that reactions apparently specific to certain pathological conditions can be induced without the specific agent, in order to establish the principle that, though the force responsible for immunity changes may not be purely physical, its effects are manifest as physical changes in a colloidal system. It is obvious that immunity reactions *in vitro* lend themselves the most readily to laboratory study, and accordingly a great deal of work has been done in the endeavour to elucidate the principles which underlie such classical examples as, for instance, agglutination and the Wassermann reaction. Great importance has recently become attached to the question of the difference of potential between organisms and the medium in which they are suspended, and to the fact that an agglutinating (but not necessarily protective) serum may reduce this potential (Northrop and De Kruif, 1922). The phrase 'agglutinating serum' is used here advisedly, as distinct from 'protective serum', because it seems clear from Shibley's

(1924) experiments that a highly protective serum may not have this charge-reducing property ; such a serum would, of course, have a low agglutination titre, and the findings suggest that the anomalies of research in this direction may yield a clue to the long-existing discrepancies in observations which show that the agglutination titre of a serum does not appear to bear any constant relation to its protective power. The Wassermann reaction has proved a fertile field of investigation by the methods applicable to the flocculation of colloidal substances. These two reactions, however, are of so much importance in themselves, and have so copious a literature, that the reader must be referred to the sections specially devoted to them. Other problems, less fully explored, in which colloidal chemistry has served to increase knowledge of the phenomena concerned, will be dealt with very briefly here under the heading of the method by which research has been prosecuted, i.e. measurement of surface tension ; flocculation of colloids ; filtration ; hydrogen-ion concentration ; viscosity ; permeability ; dialysis ; denaturation ; and adsorption.

#### *Surface Tension.*

A colloidal system is unstable until its free energy is at a minimum, and it reaches equilibrium only when no further reduction by spontaneous change is possible. Since, in bacterial suspensions, the specific surface (i.e. the ratio of surface to volume) of the organisms is very large indeed, it is probable that interfacial energies determine the dispersion or agglutination of bacterial cells in a liquid, and that interfacial tension is closely related to the factor which Buchanan (1919) calls ' cohesive force '. Mellon and his colleagues (1924) show that the cohesive power which causes bacteria to agglutinate when suspended in distilled water can apparently be reduced by sodium oleate, which lowers surface tension ; the emulsions are less readily agglutinable and the results suggest a reduction of the interfacial energies between the organisms and the surrounding medium ; these workers think that the action of sodium oleate depends upon the orientation of its molecules, which are adsorbed upon the surface of the bacteria and then lie so that the two parts of the molecule are directed, each towards the surface for which it has the greater affinity, i.e. the polar or COONa group towards the water and the fatty acid group towards the lipoidal surface of the organism, forming a transitional substance between two immiscible phases, and so making for greater stability ; they found, too, that organisms agglutinated by a low concentration of salt formed a stable suspension in a high concentration of salt, and that this latter suspension could then be diluted to the original lower concentration of salt without the occurrence of agglutination ; these more stable suspensions are, however, susceptible to the action of electrolytes, while if the treated organisms are thoroughly washed, they are again spontaneously agglutinable. Mudd and Mudd (1924) start from the principle that in a three-phase system containing bacilli and two immiscible liquids,

aqueous and organic, the bacilli occupy the position in which they most decrease the free energy of the system, whether they are drawn into either liquid or whether they remain in the interface. When the interfacial tension between the two liquids is high, the organisms remain in the interface ; when it is low, they may be found in either phase ; and when the organisms themselves have a low surface tension, e.g. acid-fast bacilli with a high content of lipoids, they are 'ingested' by the organic phase. In Ledingham's (1912) experiments, the organisms were found to congregate at the periphery of the leucocytes, or, as Mudd and Mudd would describe it, at the boundary between two immiscible phases. Ledingham thinks that surface-tension alterations between the bacterial and leucocytic membranes determine phagocytosis, particularly since he avoided mechanical effects due to spreading, by continuous shaking of the mixture of bacteria, cells and serum. Larson (1928) notes that if pellicle-forming bacteria are grown in a medium of low surface tension they are diffused through the medium, also that a staphylococcus which has a large fatty content as a result of being grown in glycerol broth, has a lower tendency to spontaneous agglutination in salt solution than the ordinary strain. These are consistent observations, but the inferences are less obvious when the same author states that the activity of diphtheria toxin can be modified by soap, that the degree of toxicity can be varied at will by altering the volume of the injected fluid, or the concentration of the soap, and that further dilution of non-toxic mixtures causes them to become toxic again.

An ingenious method of measuring surface tension by the torsion of a metal filament has been elaborated by du Noüy (1922<sup>1</sup>), who has determined many of the properties of serum relating to surface tension. If serum is examined when it is freshly separated and again when it has been allowed to stand for two hours, the surface tension is found to have fallen, because the surface-tension-reducing substances have accumulated in the top layer of the serum (Ramsden, 1903). Du Noüy calls this change the 'time-drop' (1923), and shows that it follows the ordinary law of adsorption of surface-tension-reducing substances into the liquid-air interface (1922<sup>2</sup>) ; the maximum time-drop occurs at a dilution of 1:10,000 of the serum, and du Noüy attributes this to the possibility that at this dilution the serum forms a layer of single molecules over the surface of the diluent ; at greater dilutions there may be gaps in this monomolecular layer ; and at lower dilutions the molecules may be piled up, leading in both cases to a readier evaporation of the water (1924<sup>1</sup>). He shows that in rabbits, after smallpox vaccination, the surface tension of the serum is increased, and also the time-drop, but that, in general, immunization lowers surface tension while increasing the time-drop (1924<sup>2</sup>). He also correlates the production of antibody with alterations in surface tension ; the 'time-drop' is greater (10 dynes or more) in an antiserum than in normal serum, the time-drop of which does not usually exceed eight dynes, and the increase in the time-drop runs parallel with the

development of antibody. The phenomenon is not, however, directly due to antibody, since it disappears while antibody is still present, neither is it an effect of the albumin-globulin ratio, nor of the action of antigen on antibody *in vitro* (du Noüy, 1925).

#### *Flocculation of Colloids.*

Douris and Mondain (1927) consider that pathological sera are in a state of disturbed equilibrium, which makes them behave differently from normal sera in the rate and intensity of their flocculation when distilled water is added. They also state that a drop of normal serum dried on a slide at 40° C. shows a symmetrical pattern, whereas a pathological serum does not. Du Noüy (1925) makes a similar observation to this—that an antiserum, allowed to evaporate, leaves a residue arranged differently from that of normal serum. This is perhaps due to some disturbance of the colloidal balance of the blood ; it is, of course, an accepted fact that there is often a slight increase above normal in the total protein-content of pathological sera, though the greatest change is in the albumin-globulin ratio.

The globulin increases at the expense of the albumin, according to Reitstötter (1920), though Berger (1922) does not think there is sufficient justification for the theory that albumin is turned into globulin. Berger determined over a long period the total protein and the amounts of globulin and albumin present in the serum of rabbits after an injection of horse-serum, and found that there were apparently two peaks in the total protein curve, which corresponded to the albumin and globulin maxima, the variations after a single protein injection showing marked analogies with those occurring in immunization and disease. However that may be, it is fairly certain that this albumin-globulin change is the basis of many of the reactions of the laboratory, particularly those in which attempts have been made to use the precipitation of metallic sols for diagnostic purposes. Lange's colloidal gold reaction is the classical example ; this test is not absolutely specific, since precipitation of the gold is caused by other pathological sera (Reitstötter, 1920). Joël (1925), in a monograph on the use of colloidal gold in biology and medicine, quotes some experiments of Brossa (1923) on the antagonistic behaviour of albumin and globulin in the protection of a Congo-red sol. A globulin-containing Congo-red sol is coagulated by electrolytes in much lower concentration than the pure dye solution ; sensitization increases with increasing content of globulin. Albumin protects the Congo-red-globulin sol as it protects the pure Congo-red sol, and the effect of serum varies with its content of albumin and globulin. The author thinks that this antagonism is of biological importance.

It seems possible, too, that complement fixation is a result of the aggregation of the serum-proteins, and the Wassermann reaction, regarded as a complement-fixation test, may be dependent upon the ultra-microscopic aggregation of the serum-proteins in presence of a lipoidal

extract. Silber and Tschernochwostow (1926) consider this test to be purely a question of adsorption, dependent not on a specific reaction, but on the degree of dispersion of the serum colloids ; they illustrate their point by experiments with colloidal iron, using suspensions in which the specific surface (i.e. the ratio surface : volume) of the iron is varied by the coagulating action of sodium chloride though the actual content remains the same, and show that complement is adsorbed by a finely divided sol, and not by one in which the particles are relatively large. In this connection, Dean (1911<sup>1</sup>) has reviewed the evidence for the separate identity of precipitins and complement-fixing substances. Complement fixation is demonstrable in mixtures in which no precipitate is detectable and certain antisera which form precipitates show little or no complement fixation, while in immunization the complement-fixing property appears before the property of forming a precipitate. He shows that the amount of precipitate in an antigen-antiserum mixture depends upon the relative proportions of antigen and antibody present, and that under given conditions two points can be determined, that of greatest precipitation and that of greatest complement fixation. He thinks that complement is fixed during the earliest stages of aggregation, since when visible turbidity is present, the capacity for complement fixation has largely disappeared (cf. Silber and Tschernochwostow's experiments with colloidal iron). For this reason, the rapid flocculation of an antiserum does not favour complement fixation, a slowly flocculating system being more effective. Silber and Tschernochwostow (1926) also find that the supernatant fluid from a flocculated toxin-antitoxin mixture no longer has power to fix complement, and that the time elapsing before the fixation of complement depends upon the speed of flocculation of the toxin-antitoxin system concerned. They say, however, that complement disappears earlier in a rapidly flocculating than in a slowly flocculating system, but it is to be noted that their toxin and antitoxin were mixed in optimal proportions, which favour precipitation, but do not provide the best conditions for complement fixation.

#### *Filtration.*

There is a certain amount of evidence that complement fixation is not due to adsorption, but to some condition of the serum, apart from the broad distinction to be drawn (Dean, 1911<sup>2</sup>) between the comparatively simple complement fixation of agglutination and the more complex haemolysis in which complement is necessary to complete the reaction. Muir and Browning (1909) showed that the passage of serum through a filter-candle deprives it of complement, but that the complement is not recoverable by washing the filter with salt solution, or by grinding up and extracting the substance of the filter. Adding the powdered substance of the filter to the serum does not abstract complement ; it would seem as if the structure of the serum in some way suffered a physical deformation. The filter becomes permeable to complement after a time, even though inactivated serum is used to make it so ; egg-white is quite ineffective

for this purpose. If haemolysin and complement are mixed and put in the filter, the complement is retained and the haemolysin passes through. Arloing and Langeron (1926) confirm the fact that serum loses complement by passage through a filter, and show also that haemolysin and typhoid agglutinins disappear after the fourth filtration.

#### *Hydrogen-Ion Concentration.*

The simplification of the technique for the determination of pH has made it widely applicable to biological problems and the observations recorded deal with many of the problems of immunology. For example, increasing pH lowers the potential at which agglutination occurs (Shibley, 1924); pH 6·0 to 8·3 is the effective range for haemolysis, for beyond these limits complement is not present in a form which can be reactivated by neutralization (Rockwood, 1925); the destruction of antibody by heating is delayed if it is carried out at a pH of 8·3 to 10·4 because coagulation is hindered (Silber and Schaechter, 1927); the separation of albumin from globulin with 45 per cent. ammonium sulphate is more exact if carried out at the isoelectric point of albumin pH 4·7 to 4·8 (Geill, 1926); a pH of 9·6 does not interfere with the precipitin reaction (Goodner, 1925); red blood-cells in different pH solutions are impermeable to anions and permeable to cations when sufficiently alkaline; the critical point lies between pH 8 and pH 8·3, the isoelectric point of globin (Mond, 1927); the antigenic properties of proteins increase with increasing acidity (Falk and Caulfield, 1917).

#### *Viscosity.*

Bostrom (1924) thinks that changes in hydrogen-ion concentration affect the adhesive power of cells and his conclusions seem to be borne out by some experiments of Zunz (1925) who says that in anaphylaxis the plasma becomes less alkaline, or even slightly acid, and the rate of sedimentation of the red blood-cells is lowered. A change in the rate of sedimentation may be due to an effect of the pH on the adhesive power of the cells, or, more obviously, to the greater viscosity following upon the absolute increase of red cells which is a constant phenomenon in anaphylaxis and colloidal shock (Steabben, 1925, 1926). Brodin and Richet (1921) say that hyperviscosity of the blood is a feature both of anaphylactic and peptone shock. The viscosity of the blood, which determines the peripheral resistance, is an important factor in the maintenance of blood-pressure. Waud (1928) finds that this viscosity is decreased in histamine shock, and so the blood-pressure falls. It is true that blood is incoagulable in histamine shock and both Waud and Burton-Opitz (1911) find that the viscosity of blood decreased with defibrillation; Dale and Laidlaw (1918-19), however, attribute the stagnation of the blood in the capillaries to a greater viscosity, brought about by an increase in the concentration of red blood-cells following a loss of plasma from the vessels. On the other hand, CO<sub>2</sub>, i.e. an increased hydrogen-ion concentration, increases viscosity by swelling of the red cells, and since in shock the hydrogen-ion concentration

is increased, the stagnation of blood in the capillaries may be partly a question of pH. Bostrom (1924) says that increased acidity leads to the accumulation of the blood in the cutaneous vessels, the liver and the spleen, a significant finding in relation to this question.

#### *Permeability.*

Warburg (1922) has published an exhaustive account of the literature and mathematical arguments relating to the distribution of ions between cells and plasma. He shows that the conditions which obtain are in accordance with Donnan's theory of membrane equilibria, and that volume changes in the erythrocytes are in all probability osmotic phenomena dependent upon the reaction of the system. Beyond these purely physical actions, and not yet experimentally related to them, are the selective permeability of the living cell and the variations in permeability which depend on its state of activity. Boas (1922) carried out some experiments upon plant cells with three groups of substances, saponins, narcotics and alkaloids, and found that the first group acted upon the lipoids of the cell membrane, and injured the cells by increasing their permeability. Dale and Laidlaw (1918-19) describe the increased permeability of the vessel walls in histamine shock and Petersen and his colleagues (1923) found red cells and an increased proportion of the serum-proteins in the lymph as a pathological result of the injection of certain substances. They found that tuberculin produced a similar reaction, much accentuated in the tuberculous animal and suggested that toxic material was released because of an increase of the permeability of the cells of the infected focus. It is, perhaps, this reaction which constitutes tuberculin shock ; it is certainly true that an intense focal activity follows upon the injection of colloidal substances into infected animals, and Matthes's finding in 1895, that deutero-albumose could produce the same effect as tuberculin has an obvious relationship to these later experiments. These variations in permeability are probably connected with differences in osmotic pressure.

#### *Dialysis.*

The importance of serum-globulin in immunity reactions has long been an established fact, but the older and less accurate method of separating the constituent proteins by 'salting out' has been replaced by dialysis. Electrodialysis has still further improved the method, and fractional precipitation shows that the active antibodies are in the last or pseudo-globulin fraction. Locke and Hirsch (1924) found that pseudo-globulin containing 150,000 to 200,000 units of haemolytic amboceptor per gm. could be obtained by this method, but it was not stable indefinitely, and though purer than earlier preparations was still not entirely pure. Diphtheria antitoxin has also been separated in combination with pseudo-globulin by electrodialysis (Adolf, 1924). Pseudo-globulin and euglobulin separated by this method were found to contain precipitins and also the anaphylactic substance (Doerr and Hallauer, 1926).

*Denaturation.*

Buchanan (1919) draws an analogy between the sensitization of bacteria by serum and the denaturation of protein ; the particles, protein or bacterial, when denatured, have an increased adsorptive power for ions, that is, their electric charge is decreased and they tend to agglutinate. Bacteria sensitized by heated (and, therefore, denatured) antiserum from a rabbit do not subsequently agglutinate well with the homologous serum (Jones, 1928). The theory which immediately offers itself is that the bacteria are coated with a film of serum which has lost its agglutinating properties and is itself insensitive to the immune-body of unheated antiserum ; unfortunately for this explanation, however, normal serum has no effect, i.e. it does not prevent subsequent agglutination with antiserum. Nevertheless, in both cases, the sensitized bacteria give a precipitin reaction with antirabbit-serum. The heated antiserum will fix complement in a haemolytic system, so that the physical change induced by heating provides an adsorptive surface for the mechanical disappearance of complement. Eisenberg and Volk (1902) showed that heating and the addition of acid—two methods of denaturation—considerably modified agglutinin and agglutinable substances. Manwaring and his colleagues (1928) examined the denaturation of injected protein in normal and immune dogs. They found that protein injected into normal dogs persisted for at least four months, but that within four days it had become denatured, having lost the capacity to cause anaphylaxis in a sensitized animal and also the capacity to sensitize a normal animal. They made similar experiments upon immune dogs and found that the rate of disappearance of the protein was much slower in immune dogs than in hypersensitive or normal dogs, but the toxicity of the circulating protein was maintained only for three days, after that time the protein had become denatured.

*Adsorption.*

When globulin, separated from the serum of a rabbit inoculated with vaccinia virus, is mixed with this virus, the resulting compound is non-virulent ; normal globulin also adsorbs the virus, but is then itself virulent (Henseval, 1919). But though adsorption may not in itself destroy toxin, it seems to be, at any rate, a factor in some processes of destruction. For instance, Duhamel and Thieulin (1920) found that the incubation of diphtheria toxin with a gold or silver sol reduced its activity ; in presence of a mixture of liver extract and colloidal gold, the toxin lost its lethal properties ; this may be partly an adsorption and partly an oxidation, where the one process is essential to the other, as in enzyme action. Electrically prepared gold, silver and platinum sols do not affect toxin, but ion and manganese reduce its activity, presumably by oxidation. Le Fèvre de Arric (1919) relates this to the importance of manganese in the natural oxidases. In this connection, Neill (1926) shows that tetanolysin, streptolysin and the haemolytic toxin of *B. welchii* can be inactivated by exposure to air and then reactivated by treatment with

a reducing agent; all these substances are, however, irreversibly inactivated by heat. According to Bleyer (1922) both electrical and chemical sols of iron and silver destroy agglutinins, and a 5 per cent. solution of a casein preparation called 'Caseosan' is also effective. Sachs (1919) found that complement disappeared in an inulin suspension, but not in an inulin solution; he also found that complement was destroyed by simple dilution with distilled water, and attributed the fact to an alteration of the serum-proteins—of globulin in particular. If distilled water was added and the mixture kept at 37° C., a fine precipitate formed with loss of complement; but if it was kept at 0° C., a coarser flocculation resulted and complement was not lost; this agrees with Dean's conclusions (1911<sup>1</sup>) that the finer precipitate has the greater effect on complement, but in these experiments the slower precipitation results in larger flocculi, which is not usually the case in immunity reactions. The effect of dry aluminium oxide upon botulinus toxin is noteworthy (Sommer, Sommer and Meyer, 1926). The toxin is adsorbed from solution by dry aluminium oxide, which is then filtered and washed. If the adsorption complex is directly evaporated, it is invariably non-toxic, but if the toxin is separated from the alumina by washing with dilute secondary ammonium phosphate (in, apparently, any concentration or at any pH) and then dialysed, the residue evaporated to dryness *in vacuo* is toxic, though the titre may fall from 1:100,000 to 1:1,000. Landsteiner and Stanković (1906<sup>1</sup>) showed how the protein of solutions, including those containing agglutinins, could be 'bound' by such substances as casein, fibrin and silk, also that heating, i.e. partial coagulation, of the protein solutions increased the amount adsorbed. The hæmagglutinins occurring in normal serum can be abstracted in this way, but not specific hæmagglutinins. A similar distinction is drawn by Forssman (1911). The hæmolysin produced in rabbits by heterophile antigen is specific for sheep-cells, and appears to depend for its action upon immune-body and complement, but it is not identical with the specific hæmolysin obtained by the injection of sheep-cells.

Eisler (1924) records some results which indicate the difficulty of forming any satisfactory hypothesis as to the processes concerned. Charcoal treated with antiserum does not absorb the homologous bacterial extract, but untreated charcoal does so; charcoal treated with cholera or typhoid antiserum selectively absorbs the homologous organism from an emulsion; on the other hand, after being treated with horse-serum precipitating serum, the charcoal does not react with horse-serum, but when treated with agglutinating serum for sheep-cells the latter become concentrated on the surface of the charcoal. Zanger (1905) says that saponin is absorbed within two minutes by canine red cells, which are not very sensitive to its action, but if the cells of a hen are added after an hour or so, they are hæmolyzed, showing that the first combination is reversible. Bechhold (1907) points out that substances of intense physiological action are the most affected by adsorption during ultra-filtration,

while inert proteins are not changed ; the effect is partly dependent on the size of the pores of the filter. 'Lab', a milk-coagulating enzyme, is retained by a filter which keeps back serum-albumin, but passes a filter which lets serum-albumin through. Diphtheria toxin passes unaltered through a 2·25 per cent. gelatin filter under a pressure of three atmospheres, but is retained by a 2·5 per cent. filter under a pressure of four atmospheres (cf. Muir, 1909). Dean (1912) showed that globulin is adsorbed by sensitized red cells and thinks this may be the essential part of complement fixation. He shows also that solution of guinea-pig globulin greatly increases typhoid agglutination, but this may be a purely mechanical process in which the globulin is carried down by the condensing flocculi of the agglutination. Muir and Browning (1906) found that this increased precipitation also occurred both with normal and heated guinea-pig serum, and concluded that the action was not dependent on complement.

The foregoing short survey of the researches in which immunological problems have been considered from the standpoint of colloidal chemistry sufficiently indicates that the method is a promising one. Some years ago it seemed that the specific response of the animal body to an antigen might be purely a question of chemical structure ; so far as it is possible to judge at the present time the evidence suggests that perhaps chemical structure determines specific immunity and the disturbance of physical conditions in the body makes it demonstrable. If such a possibility could be established as an experimental fact it might help to link up the anomalies of the Wassermann reaction, the production of heterophile antibody, and the true specific reactions, which at present can only be vaguely expressed as a 'disturbance of the colloidal equilibrium of the plasma proteins'.

#### REFERENCES.

ADOLF, M., 1924, *Klin. Wschr.*, **3**, 1214.  
 ARLOING, F. & LANGERON, L., 1926, *C.R. Soc. Biol.*, Paris, **95**, 843.  
 BECHHOLD, H., 1907, *Biochem. Z.*, **6**, 379.  
 BERGER, W., 1922, *Z. ges. exp. Med.*, **28**, I.  
 BLEYER, L., 1922, *Z. ImmunForsch.*, Tl. I, Orig., **33**, 478.  
 BOAS, F., 1922, *Biochem. Z.*, **129**, 144.  
 BOSTROM, E. F., 1924, *Amer. J. Physiol.*, **67**, 291.  
 BRODIN, P. & RICHET, C. FILS, 1921, *C.R. Soc. Biol.*, Paris, **84**, 298.  
 BROSSA, G. A., 1923, *Z. ImmunForsch.*, Tl. I, Orig., **37**, 211.  
 BUCHANAN, R. E., 1919, *J. Bact.*, **4**, 73.  
 BURTON-OPITZ, R., 1911, *J. Amer. Med. Ass.*, **57**, 353.  
 DALE, H. H. & LAIDLAW, P. P., 1918-19, *J. Physiol.*, **52**, 355.  
 DEAN, H. R., 1911<sup>1</sup>, *Proc. R. Soc. Med.*, **5** (Sect. Path.), 62 ; 1911<sup>2</sup>, *Proc. Roy. Soc.*, **84**, B, 416 ; 1912, *J. Hyg., Camb.*, **12**, 259.  
 DOERR, R. & HALLAUEER, C., 1926, *Z. ImmunForsch.*, **47**, 363.  
 DOURIS, R. & MONDAIN, C., 1927, *C.R. Acad. Sci.*, Paris, **185**, 232.  
 DUHAMEL, B. G. & THIEULIN, R., 1920, *C.R. Soc. Biol.*, Paris, **88**, 292.  
 EISENBERG, P. & VOLK, R., 1902, *Z. Hyg. InfektKr.*, **40**, 155.  
 EISLER, M., 1924, *Biochem. Z.*, **150**, 350.  
 FALK, I. S. & CAULFIELD, M. F., 1923, *J. Immunol.*, **8**, 239.  
 FORSSMAN, J., 1911, *Biochem. Z.*, **87**, 78.  
 GEILL, T., 1926, *C.R. Soc. Biol.*, Paris, **95**, 1101.  
 GOODNER, K., 1925, *J. Infect. Dis.*, **37**, 285.

THE BEARING OF COLLOIDAL CHEMISTRY  
UPON SOME IMMUNITY REACTIONS

HENSEVAL, M., 1919, *C.R. Soc. Biol.*, Paris, **82**, 1071.  
JOËL, E., 1925, *Das Kolloide Gold in Biologie u. Medizin* (Leipzig : Akademische Verlagsgesellschaft), p. 87.  
JONES, F. S., 1928, *J. Exp. Med.*, **47**, 245.  
LANDSTEINER, K. & STANKOVIĆ, R., 1906, *Zbl. Bakter.*, Abt. I, Orig., **41**, 108.  
LARSON, W. P., 1928, *The Newer Knowledge of Bacteriology and Immunology* (Chicago : University of Chicago Press), p. 179.  
LEDINGHAM, J. C. G., 1912, *J. Hyg.*, Camb., **12**, 320.  
LE FÈVRE DE ARRIC, 1919, *C.R. Soc. Biol.*, Paris, **82**, 1143.  
LOCKE, A. & HIRSCH, E. F., 1924, *J. Infect. Dis.*, **35**, 519.  
MANWARING, W. H. *et alii*, 1928, *J. Immunol.*, **15**, 351.  
MATTHES, M., 1895, *Deut. Arch. klin. Med.*, **54**, 39.  
MELLON, R. R. *et alii*, 1924, *J. Immunol.*, **9**, 365.  
MOND, R., 1927, *Klin. Wschr.*, **6**, 1432.  
MUDD, S. & MUDD, E. B. H., 1924, *J. Exp. Med.*, **40**, 647.  
MUIR, R. & BROWNING, C. H., 1906, *J. Hyg.*, **6**, 20 ; 1909, *J. Path. Bact.*, **13**, 232.  
NEILL, J. M., 1926, *J. Exp. Med.*, **44**, 215, 227.  
NEILL, J. M. & MALLORY, T. B., 1926, *J. Exp. Med.*, **42**, 241.  
NORTHROP, J. H. & DE KRUIF, P. H., 1922, *J. Gen. Physiol.*, **4**, 629, 639, 655.  
DU NOÜY, P. L., 1922<sup>1</sup>, *C.R. Acad. Sci.*, Paris, **174**, 962 ; 1922<sup>2</sup>, *J. Exp. Med.*, **35**, 575, 707 ; 1923, *ibid.*, **37**, 659 ; 1924<sup>1</sup>, *ibid.*, **39**, 717 ; 1924<sup>2</sup>, *ibid.*, **40**, 29 ; 1925, *ibid.*, **41**, 779.  
DU NOÜY, P. L. & BAKER, L. E., 1925, *J. Exp. Med.*, **42**, 9.  
PETERSEN, W. F. *et alii*, 1923, *J. Immunol.*, **8**, 349, 361, 377, 387.  
RAMSDEN, W., 1903, *Proc. Roy. Soc.*, **72**, 156.  
REITSTÖTTER, J., 1920, *Z. ImmunForsch.*, Tl. I, Orig., **30**, 468, 507.  
ROCKWOOD, R., 1925, *J. Immunol.*, **10**, 741.  
SACHS, H., 1919, *Kolloid Z.*, **24**, 113.  
SHIBLEY, G. S., 1924, *J. Exp. Med.*, **40**, 453.  
SILBER, L. & SCHAECHTER, E. P., 1927, *Z. ImmunForsch.*, **53**, 263.  
SILBER, L. & TSCHERNOCHWOSTOW, W., 1926, *Z. ImmunForsch.*, **48**, 472.  
SOMMER, E. W., SOMMER, H. & MEYER, K. F., 1926, *J. Infect. Dis.*, **39**, 345.  
STEABBEN, D. B., 1925, *Brit. J. Exp. Path.*, **6**, 1 ; 1926, *ibid.*, **7**, 141.  
WARBURG, E. J., 1922, *Biochem. J.*, **16**, 153.  
WAUD, R. A., 1928, *Amer. J. Physiol.*, **84**, 563.  
ZANGGER, H., 1905, *C.R. Soc. Biol.*, Paris, **58**, 589 ; **59**, 664.  
ZUNZ, E., 1925, *Bull. Acad. Méd. Belg.*, 5<sup>e</sup> sér., **5**, 334.

## CHAPTER XVII. CHEMOTHERAPY.

. By C. H. BROWNING (UNIVERSITY OF GLASGOW).

### Introduction.

CHEMOTHERAPY is a term coined by Ehrlich to indicate the treatment of infections by compounds of known chemical constitution which effect cure by leading to destruction of the pathogenic organisms or their products. Thus a contrast is implied between chemotherapeutic substances and the class of antibodies, which represent highly complex, specific products of the biological reaction to the infecting organisms. Chemotherapeutic agents may be supposed either to act directly on the parasites so as to cause their death, or, on the other hand, their action may be an indirect one, due to stimulation of the natural defensive powers of the tissues. There is evidence that both these mechanisms may operate in different cases. If the vitality of the micro-organisms is lowered by the direct action of the chemotherapeutic agent, then of course the cellular and humoral tissue defences will be enabled to come into play ; in this way a co-operated action may take place. Further, constituents of the organisms which are destroyed, have the capacity for acting as antigens, so that an immunity reaction may also be involved.

It is clear that the difficulty of effecting direct chemotherapeutic action will be much greater when the infection is generalized or situated in the deep tissues, e.g. in the central nervous system, than when it is localized and relatively accessible. Under the latter circumstances the therapeutic problem may be solved by applying an agent which is more destructive to the organisms than to the tissues. But if the infection is general or deeply seated the chemotherapeutic agent must circulate in the blood or lymph in order to reach the organisms, and when this is attained the body as a whole is exposed to its toxic action ; also the substance is liable to be excreted or destroyed or inactivated by fixation in the tissues. When the parasites are situated in avascular or necrotic tissues or actually within cells, the difficulties will be further enhanced. In all cases the toxicity of a substance for the host is the limiting factor which determines whether or not it can be employed as a chemotherapeutic agent. Wright (1927) has summed up the conditions which may account for failure in chemotherapeutic action.

So far, chemotherapy has been most effective in general infections with protozoa and spirochætes, the oldest established chemotherapeutic agents being, of course, mercury and its salts in syphilis, quinine in malaria and ipecacuanha in amœbic dysentery. The only general bacterial

infection which has been cured by such means is that due to pneumococci in mice, which responds to the administration of ethylhydrocupreine (optoquine) as Morgenroth and Levy (1911) showed. Various local bacterial infections are amenable to treatment by a number of chemical substances ; so far, however, no chemical compound is known which can influence within the body the pathogenic action of an exotoxin in a manner comparable to that of the specific antitoxin. The first attempt at chemotherapy on experimental lines appears to have been made by Koch (1881), who treated guinea-pigs infected with anthrax bacilli by subcutaneous injections of a solution of mercuric chloride, this substance being selected because of its powerful bactericidal action on the bacilli of anthrax *in vitro*. In order to ensure as far as possible adequate diffusion of the mercury salt, three to five daily doses were given before inoculation and one on the same day and on that following. The animals so treated died of anthrax within 48 hours after inoculation. These experiments showed that an amount of mercuric chloride in proportion to the animal's body-weight, which if present in broth *in vitro* would have completely prevented growth of the organisms, was entirely without effect *in vivo*. This lack of agreement, which has been repeatedly observed in such work, illustrates one of the chief difficulties met with. For instance, Hata (1910) found that in mice infected with *Spirochæta recurrentis* no effect followed the injection subcutaneously of a dose of methylene blue which, reckoned according to the animal's body-weight, was about 500 times that lethal for the parasites *in vitro*.

The first effective synthetic substance discovered as the result of purposive investigation was Weinberg's trypan-red, with which Ehrlich and Shiga (1904) cured the certainly fatal experimental infection produced in mice by *Trypanosoma equinum* (the cause of Mal de Caderas). From this beginning the development of chemotherapy as a branch of research received its chief impulse through Ehrlich. Ehrlich and Hata's use of salvarsan in syphilis represents so far the most effective application in human therapy. The recent work of Walburn with metallic salts indicates the therapeutic possibilities of substances which almost certainly do not produce their effects by exerting any direct harmful action on the infecting organisms, as appears also to be the case with iodides in syphilis and actinomycosis.

In the present chapter the chemotherapy of infections due to trypanosomes and spirochaetes will be dealt with first, since the fullest observations have been made with these. Metazoal infections will not be considered.

#### Infections with Trypanosomes.

Certain trypanosome infections, e.g. those with *Trypanosoma brucei*, *T. equinum*, *T. equiperdum*, &c., when adapted to small rodents such as mice and rats by repeated passages through these hosts, form one of the most suitable objects for chemotherapeutic investigation, as in untreated animals the parasites increase progressively in the blood, and death occurs

invariably within a few days ; also, after inoculation with numbers of parasites which may vary within fairly wide limits the length of survival for a given species of host and parasite is remarkably constant. An effective therapeutic agent in an adequate dose leads to permanent sterilization of the body. The parasites disappear from the blood more or less rapidly according to the nature of the substance employed. They show various morphological changes indicative of degeneration (see Steffan, 1922 ; Sei, 1923), and they are finally disposed of, partly by phagocytosis (see Laveran and Mesnil, 1912) and partly by undergoing solution (see Moldovan, 1914).

The effectiveness of treatment may depend greatly on the stage of the infection at which the drug is given ; generally sterilization is more readily produced when the therapeutic agent is administered early. Thus, a substance of low activity, such as parafuchsine, acts best when the tissues are saturated with it before inoculation, as may be effected by continuous feeding with the dye. But there are drugs, e.g. certain of the styryls, which act equally well at an advanced stage (Browning, Cohen, Ellingworth and Gulbransen, 1929). It should be noted that an immunity reaction follows destruction of the trypanosomes under the influence of the therapeutic agent, and this is likely to be greater when the parasites are present in considerable numbers than when they are very scanty (Braun and Teichmann, 1912). In certain cases the parasites may increase for several days after the administration of the drug and yet cure may result. This phenomenon is not to be accounted for entirely by slow resorption of the drug, but points to the ultimate intervention of antibodies. Where an effect is produced short of complete sterilization, the parasites may disappear from the blood temporarily ; but after an interval relapse occurs and the infection may then run its course as in an untreated animal, or the course may be chronic, the trypanosomes fluctuating in numbers for a period and death finally occurring when they are abundant. A substance which has no observable effect on the course of the infection may, nevertheless, influence the parasites. This is seen in the case of potassium hexatantalate ; when infected mice are treated with it in successive passages the trypanosomes finally fail to cause infection on inoculation into a further animal (Morgenroth and Rosenthal, 1911).

Relapses in mice are notably difficult to cure. Some trypanosomes, e.g. *T. gambiense*, even in untreated animals cause a relapsing type of infection which is not readily cured. Regarding the explanation of relapses after treatment, it is evident that a proportion of the parasites are more resistant than the majority and that the drug merely leads to depression of their activity without their being actually killed. But it is not possible to determine whether the resistance is an inherent property of certain individual trypanosomes or whether it is due to their being in situations in which they are inaccessible to the trypanocidal agent. In the case of *T. cruzi* an intracellular stage exists in which the parasites may be protected, and a similar occurrence has recently been described in

other species (Peruzzi, 1928). As will be seen, the trypanosomes which reappear in the blood are resistant to the antibodies developed as a result of treatment of the original infection (p. 510).

With regard to dosage, the efficacy of a substance is conveniently measured by the ratio between the smallest amount which when administered in one dose will effect cure and the largest amount which the host will tolerate. In the case of Bayer-205 in mice infected with some strains of *T. brucei*, this ratio is 1 : 150 to 1 : 300, which is the most favourable so far obtained for any animal and any substance. It should be noted that the minimum curative dose is not always readily ascertainable, since certain substances show a wide range between the dose which cures some individuals and that which fails in others of the same species.

The other method of treatment is by fractional dosage, where (without waiting for a relapse) the attempt is made to effect sterilization by means of a series of doses each of which is insufficient by itself; cure may then result, probably apart from mere accumulation of the drug in the body (Laveran, 1905 ; Franke, 1905). This method, of course, is one extensively used in human treatment, where the danger of poisoning must be avoided, and therefore the individual doses are small. In the treatment of bird malaria by plasmoquine and other substances a series of doses appears to be essential in order to effect cure. Variations in the route of administration, whether subcutaneous, intraperitoneal, intravenous or by the alimentary tract, may influence therapeutic action by affecting the absorption and consequently the concentration of the substance in circulation. The concentration in which the drug comes into contact with the tissues may also have an influence, since Kroó and Manó (1927) found that a given dose of neosalvarsan administered intravenously in dilute solution (1 : 300) produced cure in a greater proportion of mice infected with *Sp. recurrentis* than when administered in higher concentration (1 : 75 ; 1 : 30). A similar effect of dilution was also demonstrated for mice infected with *T. brucei*; but with trypaflavine the action was independent of the concentration. When a relatively insoluble drug is introduced, for instance, intramuscularly, a 'depot' is produced which serves to maintain a certain concentration of the drug in the circulation over a considerable period. Thus, Hata (1910) showed that an intramuscular injection of salvarsan protected fowls against infection with spirochaetes for 30 days, whereas animals were fully susceptible to inoculation six days after an intravenous injection. The toxicity of the drug for the host may also depend on the route of introduction. In the case of drugs used in human therapy it is necessary to standardize the toxicity accurately, especially when, as in the case of salvarsan products, this is affected by factors in the preparation which are difficult to control (see Durham, Gaddum and Marchal, 1929).

Trypanocidal properties have been found in a variety of chemical classes, e.g. compounds of arsenic, antimony and bismuth, derivatives of quinine, organic basic compounds of the types of triphenylmethane dyes

(parafuchsine, tryparosan), pyronines, oxazines, aminoacridine (trypaflavine) and aminostyryl quinoline, also acid bis-azo dyes (trypan-red, trypan-blue) and the related colourless compound Bayer-205. Relationships between chemical constitution and therapeutic action which have been established in one group do not necessarily hold in another. Thus, analogies are not yet sufficiently extensive to enable one to predict, apart from actual trial, what chemical constitution is likely to be accompanied by therapeutic action ; this is well exemplified by the investigations of King along with Balaban, Durham, Hewitt, Marchal and Murch on arsenical compounds (1924, 1925, 1926, 1927) and of Fourneau, Tréfouel and Vallée (1924) and Bauer and Becker (1928) on the Bayer-205 group.

A series of phenomena have been discovered in connection with the chemotherapy of trypanosome infections which may be of more general occurrence, but which possibly cannot be detected with the same ease in other conditions. As important principles are involved in these, they will be considered in greater detail.

#### *Drug-resistance.*

When a small dose of a therapeutic agent is given to an inoculated animal such as a mouse the course of the infection may scarcely be influenced. If the trypanosomes are then transferred to a fresh mouse the same absence of effect may attend a slightly larger dose than that previously administered. By proceeding in this way the parasites may be so modified after treatment in a series of passages that finally the trypanosomes will withstand the largest doses which are tolerated by the host, corresponding to many times that curative for the infection with the original parasites. The strain has become drug-resistant as Franke and Roehl first found (see Ehrlich, 1907 ; Browning, 1907, 1908). Minor degrees of resistance are manifested by a delay in the rate of disappearance of parasites from the blood following administration of the therapeutic agent (Morgenroth and Rosenthal, 1911). Such resistant strains were originally developed by administering to begin with a dose which had some therapeutic action but which led to relapse ; then the relapse parasites were transferred to a fresh animal which was similarly treated, and so on, the doses of the drug being gradually increased in successive passages. The chief objection to this method is that the strain which results, in addition to being drug-resistant, is also serum-fast (p. 510 ; see Lewy and Gurewitsch, 1926). The rate at which drug-resistance is developed may vary widely and depends on the nature and dosage of the therapeutic agent and probably also on the individual host. In the case of trypaflavine the first relapse after administering a non-sterilizing dose of this substance to infected mice shows resistance. It must not be supposed, however, that repeated non-sterilizing doses of any chemotherapeutic agent when administered to the same animal will inevitably lead to an infection resistant to the drug used ; in fact, cure may result finally under these conditions, as was shown with styryl quinoline compounds by

Browning and Gulbransen (1928<sup>2</sup>). A similar result has also been observed where treatment in successive passages was carried out with Bayer-205, together with another drug such as arsacetin or trypaflavine (Leupold, 1928). Very small doses of a therapeutic agent are said at times to act as a stimulant to the parasites and so to intensify the infection.

Drug-resistance is in some instances highly specific ; a parafuchsine-fast strain of *T. brucei* is normal in its resistance to arsenicals, trypan-blue and trypaflavine, in fact, to chemotherapeutic agents of other chemical types (Browning, 1908). Again, acquired resistance to one compound is usually accompanied by resistance to chemically allied substances only, e.g. a strain which has been rendered resistant to trypan-blue is also resistant to Bayer-205, notwithstanding more than 200 times the curative dose of the latter for the original strain (Leupold, 1924). This result is the more remarkable in view of the fact that trypan-blue has a relatively weak therapeutic action. On the other hand, certain substances rapidly develop resistant strains which are fast also to other types of chemotherapeutic agents (Ehrlich, 1909<sup>3</sup> ; Kudicke, 1912) ; this is characteristically the effect of substances of the pyronin and acridine series (Ehrlich, 1909<sup>3</sup>), e.g. trypaflavine, to which an orthoquinoid structure is attributed. Conversely arsenic-resistant strains are resistant to trypaflavine also. As might be expected from what has been stated above, it is possible to render trypanosomes resistant to the various classes of therapeutic agents by successive treatment with members of each group ; thus, a strain may finally be resistant to parafuchsine, trypan-blue and arsacetin.

Varying grades of resistance to therapeutic substances of a single chemical type are met with. A strain rendered maximally resistant to arsacetin shows little or no increase in resistance to arsenophenylglycine ; but one rendered resistant by treatment with the latter compound is also highly resistant to arsacetin (Roehl, 1909<sup>2</sup>). By successive treatment with arsacetin, arsenophenylglycine and arsenious acid strains of four grades of resistance to the arsenical group have been produced (Ehrlich, 1909<sup>1 & 2</sup> ; Gonder, 1912<sup>2</sup>) ; but resistance to arsenious acid is developed only with the greatest difficulty. It does not follow, however, that a substance which influences trypanosomes rendered resistant to other members of the same chemical group will, therefore, be an effective therapeutic agent toward the original trypanosome strain. Thus, an arsacetin-fast strain of *T. brucei* responds to treatment with tartar emetic, which belongs to the arsenical group ; yet it is impossible to sterilize completely either an infection with the original strain or with the arsacetin-fast strain by means of the antimonial. Similarly, arsenious acid has only a weak therapeutic effect.

A substance which does not influence the course of the infection in the highest tolerated doses may, nevertheless, be capable of developing resistance to a trypanocidal agent ; this is the case with potassium hexatantalate, which develops resistance to tartar emetic (Morgenroth and Rosenthal, 1911).

The behaviour of resistant strains to treatment with the corresponding drug may vary with the species of the host (Breinl and Nierenstein, 1909; Mesnil and Brimont, 1908). For instance, a strain resistant to atoxyl in the mouse may not be resistant when transferred to rats, but on retransferece to mice the resistance is again apparent. It must be remembered, however, that resistance is a relative term and other drug-fast strains have manifested their resistance also when transferred to a different species of host (Ehrlich, 1907; Mesnil and Brimont, 1908; Roehl, 1909<sup>1</sup>).

In some cases the resistance of the trypanosomes can be demonstrated *in vitro*, the concentration of the drug required to immobilize resistant parasites being much greater than that for the normal strain, as Mesnil and Brimont (1908) showed with a strain resistant to tartar emetic. This strain required a concentration 300 times greater than sufficed to immobilize the normal strain immediately. Ehrlich (1909<sup>2</sup>) and Neven (1909) found in the case of arsenic-resistant strains also, that these remained actively motile *in vitro* for 25 minutes in the presence of 1 : 100,000 of an arsenical drug, paraoxyphenylarsenious oxide, which in a concentration of 1 : 2,000,000 immobilized the original strain in 8 minutes. On the other hand, resistance to parafuchsine cannot be demonstrated in this way, both normal and resistant parasites being readily killed by this substance *in vitro* (Neven, 1909).

Resistance may also be demonstrated by vital staining, thus Roehl and Gulbransen observed that living trypanosomes of normal resistance became stained *in vitro* with the orthoquinoid dye triaminophenazoxonium chloride, whereas the arsenic-resistant strains under similar conditions remained colourless (see Ehrlich, 1909<sup>2</sup>; Gonder, 1912<sup>2</sup>). The acquisition of resistance especially to orthoquinoid substances (pyronin, trypaflavine) is accompanied also by a striking morphological change in the parasites, the blepharoplast disappearing (Werbitzki, 1910; Kudicke, 1911). Robertson (1929) has found that trypaflavine when acting *in vitro* on cultures of the protozoon *Bodo caudatus* causes a similar effect in a proportion of the organisms. Trypanosomes of a strain which has been rendered resistant to arsenic or trypan-blue do not lose their blepharoplast, however, under the influence of trypaflavine; but Bayer-205-resistant trypanosomes, like the normal strain, lose the blepharoplast in response to treatment with the acridine compound (Leupold, 1925).

After a drug-fast strain has been passed through a long series of untreated animals over a period of many months or even several years, it may still possess maximal resistance; also if the strain is devoid of blepharoplast this character is retained. On the other hand, it has been recorded that the resistance disappeared after repeated passages through normal animals. The latter result may be due simply to a gradual change of susceptibility or it may be explained on the basis of the strain consisting to begin with of a mixture of resistant and non-resistant parasites from which the former have become eliminated in course of time. In support of the latter view is the fact that when untreated animals were infected

with artificial mixtures of a drug-resistant strain along with the original strain from which it was derived, the former component became eliminated after repeated passages through untreated mice (Oehler, 1914). The drug-fast strains may therefore have suffered some loss of proliferative power, as may be the case also with relapse strains (p. 510). But where the strains are of equal virulence either one or the other may suffer elimination (Teichmann, 1916). According to Laveran and Roudsky (1912), however, when an artificial mixture of the blepharoplast-free strain with the original strain is passed repeatedly through mice, the blepharoplast-bearing trypanosomes are soon eliminated ; this result has been obtained with other species in addition to *T. brucei*.

Of great practical importance for therapy is the observation, which has been repeatedly made, that resistance to one group of therapeutic agents may be lost when the trypanosomes are rendered resistant to another drug ; as an example, Leupold (1924) observed that a strain of *T. brucei* which had been rendered fast by means of pyronin and, therefore, was resistant to arsacetin, became susceptible to the latter substance as a result of repeated treatment with Bayer-205. Gonder (1911) recorded that the passage of drug-fast *T. lewisi* through an invertebrate host, the rat louse, led to loss of resistance to arsenicals, but this result was not obtained by passage through rat fleas (Reichenow and Regendanz, 1927) ; Duke (1927) seems also to have found no such change when an atoxyl-fast strain of *T. brucei* derived from monkeys was passed through *Glossina palpalis*. In all these experiments the drug-resistance of the parasites was not of a high order to begin with.

Apart from experimentally produced drug-resistance, infections with different strains of the same trypanosomes may vary greatly in their susceptibility to cure by particular drugs. This may result from passage of the parasite through hosts of different species ; Ehrlich's 'ferox' strain of *T. brucei* arose in this way. Drug-resistance has also been found to develop in the course of repeated passages of a strain through untreated mice alone (Morgenroth, 1924) ; this has been confirmed by Browning and Gulbransen (unpublished), but on what the selective effect may depend is obscure. Different species of trypanosomes under natural conditions also cause infections which vary greatly in their responsiveness to chemotherapeutic agents ; the susceptibility of *T. rhodesiense* to Bayer-205 and the resistance of *T. congolense* to the same drug are striking examples.

#### *Chemoflexion.*

If a certainly curative dose of such a chemotherapeutic agent as salvarsan is given to a mouse heavily infected with trypanosomes and its blood is withdrawn after a short interval (one hour) and injected into another mouse, the infection in the latter may show marked resistance to the same drug. This phenomenon of chemoflexion was discovered by Morgenroth (1924) and Murata ; its occurrence has been confirmed by Browning and Gulbransen using arsacetin solely for treatment

(unpublished). On further passages through untreated animals the resistance quickly vanishes. Chemoflexion would appear to be the precursor of the more permanent form of drug-resistance already described; but the remarkable feature is the rapidity of its development in parasites which if not removed from the original host would certainly be destroyed by the treatment.

#### *Combined Therapy.*

The specific nature of resistance to different types of chemotherapeutic agents suggests strongly that each has its special point of attack in the parasite. It might, therefore, be expected that treatment with drugs of several different types would yield better results than with one alone; this has been realized in certain cases. Laveran (1904, 1905) showed that trypan-red together with arsenious acid sterilized rats, dogs and monkeys infected with *T. gambiense*, whereas neither substance by itself effected cure (see also Tsuzuki, 1911). Morgenroth and Freund (1924; see also Freund, 1925) found that by administering a non-curative dose of Bayer-205 to mice at the time of inoculation with *T. brucei* cure of the developed infection was later effected by tartrate of antimony, whereas relapse always followed the same dose of the latter alone. A similar effect was got when the trypanosomes from the animal which had received Bayer-205 were transferred to a fresh mouse, the latter being treated with the antimonial. These results were attributed to an *antimutative action* of Bayer-205, the development of a relapse strain (p. 510) being prevented; but this action occurs only with certain strains of the parasites (Kritschewski and Kaganova, 1929). The result may be due simply to a weakening of the parasites by the first treatment.

#### *Therapeutic Interference.*

The presence of a therapeutic agent in an ineffective concentration in the body of an infected animal may inhibit the action of an otherwise curative dose of another substance. Browning and Gulbransen (1922, 1927) originally demonstrated that trypaflavine failed to influence therapeutically a parafuchsine-fast strain of *T. brucei* in mice whose tissues contained parafuchsine—consequent on feeding with that substance—whereas in animals fed on normal diet this strain was susceptible to trypaflavine. Schnitzer (1926) with Rosenberg (1926, 1927) and with Silberstein (1927<sup>1,2 & 3</sup>) showed that the phenomenon was a general one and could be demonstrated with normal strains of trypanosomes, although both the individual host and the species of the parasite affect the result, according to Brussin (1929). A minute dose of parafuchsine injected some hours before a dose of arsacetin, salvarsan, tryparosan or trypaflavine prevented any of the latter from exerting their usual therapeutic effect. It is to be noted that the interfering substance may belong either to the same class as that interfered with, in the sense of drug-resistance, or they may belong to different classes. All therapeutic substances do not appear capable of

producing interference. Trypan-blue, a bis-azo dye, is effective ; tryparosan, again, which is closely related to parafuchsine, does not produce interference. A small dose of parafuchsine does not interfere with the action of a large dose of the same dye administered afterwards. Voegtlin, Dyer and Miller (1924) showed that compounds containing an SH-group were able to a certain extent to inhibit the trypanocidal action of arsenicals *in vivo* and *in vitro*. According to Schnitzer and Silberstein (1927<sup>3</sup>) one is dealing here with the interference phenomenon.

#### *Immunity following Treatment with Chemotherapeutic Agents : Serum-resistance.*

Trypanosomes act as antigens and the production of powerful antisera with protective properties by means of injections of killed parasites has been described (see Braun and Teichmann, 1912). But failure has frequently attended attempts to immunize in this way, e.g. the experiments of Browning (1927) and Gulbransen and of others in which blood containing abundant trypanosomes killed by the addition of water were used. On the other hand, if mice infected with trypanosomes are cured by a chemotherapeutic agent, immunity results (Ehrlich and Shiga, 1904). This is shown by inoculating the animals a few days up to several weeks or months later with the same strain ; the second infection may fail to develop, but more commonly it develops after a prolonged incubation period ; also the second infection may run a prolonged course. That these effects are not due to traces of unexcreted drug can be shown by using a rapidly excreted substance, such as one of the arsenicals, and by inoculating animals which have received a similar dose but which have not been infected beforehand ; the infection develops in the latter as in normal controls. (It should be noted that owing to the long persistence of Bayer-205 in the body, this substance is not suitable for such experiments.) The immunity reaction may be marked even where multiplication of the parasites has been very slight or possibly absent altogether (Terry, 1911 ; Kudicke, 1912). The grade of immunity appears to be independent of the particular drug used (Neumann, 1911), but depends on the strain of parasites (Browning, 1927, with Gulbransen). Thus a prolonged incubation on reinoculation is evidence of immunity developed as a result of destruction of the parasites of the original infection under the influence of the drug.

If the cured animals are reinoculated with trypanosomes of the same origin but derived from the relapse in an insufficiently treated animal, i.e. a 'relapse strain', there is then no prolongation of the incubation period ; the same result follows when one employs for the second inoculation the parasites developing in a cured animal after reinoculation with the original strain. Therefore, the relapse strain is immunologically different from the original parasites ; this difference consists in the parasites possessing *serum-resistance*, i.e. they are resistant to the antibodies developed after treatment of the original infection, as

Ehrlich (1909<sup>2</sup>) and his co-workers first showed. The relapse strain when passed repeatedly through untreated mice retains its new serological characters ; thus, animals infected with this strain and cured by a drug show immunity on reinoculation with the same relapse strain, but develop an infection immediately after inoculation with the original strain. When treatment causes merely a protraction of the course of the disease, without complete disappearance of parasites from the blood-stream, they may assume the relapse character (Silberstein, 1927). Ehrlich with Roehl and Gulbransen (1909) showed that a relapse strain can be developed also *in vitro* by mixing the original strain of parasites with serum from a cured animal and after a few minutes' centrifuging and inoculating the treated trypanosomes into a normal mouse. Provided the concentration of serum used for treating the parasites has not been sufficient to lead to death of the parasites, an infection develops which has the character of a relapse strain. In this connection it may be noted that in small animals infected with trypanosomes normal human serum, unlike the serum of other species, has a definite therapeutic action except in the case of *T. gambiense* (Laveran, 1902). Trypanosomes which are susceptible *in vivo* are also killed *in vitro* at 37° C. by human serum in high dilutions (Yorke, Adams and Murgatroyd, 1930). The serum-resistant character of *T. gambiense* persists as a rule for many years when the strain has been passed through animals.

The phenomena of serum-resistance have been extensively investigated and it has been found that a number of strains may be distinguished serologically when an animal repeatedly receives insufficient treatment, so that a series of relapses occurs. In general the first relapse strain tends to be serologically identical in different individuals, although this is not always the case (Neumann, 1911) ; thereafter the order in which the different strains appear is irregular. At one point the original strain may come up in a relapse ; again, the parasites in a relapse may have the serological characters of two strains ; these are referred to by Ehrlich (1909<sup>2</sup>) as 'binios' in contrast to 'unios' which only possess the serological characters of a single strain. In order to destroy the infectivity of a binio it is necessary to treat it *in vitro* with high concentrations of antisera to each of its two components ; after treatment with an antiserum to only one of these there results on inoculation a strain with the serological characters of a unio, which is then susceptible only to the other antiserum. A similar result is obtained when the binio is inoculated into cured animals which had been originally infected with the respective unios. The antibody in the serum which causes trypanocidal action is considered by Rosenthal (1913) to be different from that which leads to the development of parasites of the relapse type, since the former property is the more thermo-stable. This investigator and also Ritz (1914, 1916) have shown that the serological characters of relapse strains are exceedingly complicated and the details cannot be gone into further here.

Schnitzer and Silberstein (1928) have demonstrated very strikingly by the following experiment the part which acquired immunity may play in leading to cure of trypanosome infections. Mice were inoculated subcutaneously with an original strain of *T. brucei*, and 24 to 48 hours later were reinoculated with the same strain which had been rendered drug-resistant, e.g. to arsenicals, without being converted into a relapse type. When parasites were swarming in the blood a dose of an arsenical drug was given sufficient to cure infection with the susceptible parasites. The result was complete cure ; this must depend on immunity developed in response to the action of the arsenical on the original infection leading to the destruction of the drug-fast parasites. The co-operation of the immunity reaction in conducting to cure is also apparent where a drug has been administered which exerts a prolonged prophylactic action, and, after a considerable interval, on inoculation parasites appear in the blood and then disappear permanently (Browning, 1927, with Gulbransen).

Rieckenberg (1917) has demonstrated the difference in the reaction of the original and the relapse strains to antibodies in the following way. When a drop of uncoagulated blood from a mouse cured of infection with the original strain, which therefore contains the corresponding antibody, is mixed with a drop of citrated broth and blood containing the homologous parasites the surface of the latter quickly becomes covered with blood-platelets ; but if the trypanosomes belong to a relapse strain, then no such adhesion of platelets occurs under the influence of antibody to the original strain. This observation has been confirmed by others (Brussin and Beletzky, 1925), and it has been found with living *Sp. recurrentis* that bacteria introduced into the mixture show adhesion to the spirochaetes under similar conditions, just as the platelets do (Krantz, 1926).

Chronic trypanosome infections, for example that due to *T. brucei* in the rabbit, follow a relapsing course and this condition along with the pathogenic effects presents a type of disease closely similar to many human cases of trypanosomiasis. The parasites of the relapses consist of a series of serologically varying strains, and in the later stages the blood contains at one time a mixture of many strains (Ritz, 1914, 1916).

*Leishmaniasis.* The clinical observation that antimonials act on diseases due to Leishmania organisms has been confirmed by Roehl (1929) in the experimental infection of hamsters with the parasites of kala-azar. The efficacy of treatment was tested by examining the number of parasites in liver pulp obtained by puncture *in vivo*.

#### Infections with Spirochaetes.

Diseases due to spirochaetes resemble those caused by trypanosomes in many respects as regards their behaviour toward chemotherapeutic agents. On the other hand, there are certain differences, which will be dealt with in more detail. Some spirochaetes cause infections in which the parasites are present chiefly in the blood, e.g. those of relapsing fever,

fowl spirochætosis and *Sp. muris* in the mouse and rat ; the two former when not acutely fatal undergo spontaneous sterilization finally, but the last, although practically not pathogenic, appears to persist indefinitely in small numbers in the host's blood. Where relapses occur spontaneously, as in untreated infections with *Sp. recurrentis*, the relapse organisms have become serum-fast, being resistant to the antibodies developed in the blood after the original attack, and this character may be retained after repeated passages through normal animals (Levaditi and Roché, 1907 ; Manteufel, 1908 ; Jancsó, 1918). A series of serologically different strains has been stated to occur in the relapses and also when the parasites infect different species, but repeated passages through mice cause these differences to disappear ultimately (Kudicke, Feldt and Collier, 1924). In the case of the Indian relapsing fever spirochæte, Cunningham (1925) found that the strain derived from the first relapse remained serologically constant on passage through animals ; but when an infection with it was allowed to relapse the organism which reappeared had the serological characters of the original strain ; so that there are only two serological types of this parasite. Meleney (1928), working with relapsing fever spirochætes of Chinese origin which gave rise to several relapses in splenectomized squirrels, has confirmed this result, but by examining the spirochætes isolated from animals in successive passages he has detected also certain differences between the strains developing in alternate attacks. *Treponema pallidum* in man, monkeys and rabbits invades the tissues widely and is scanty in the blood ; also, the chronic infections to which it gives rise have practically no tendency to become sterilized spontaneously.

Such differences in the distribution of the various organisms, apart from the question of their virulence, might be expected to influence markedly the susceptibility to treatment. It has been found, however, that even in the infection due to *Spirochæta recurrentis* in mice, after treatment with salvarsan scanty organisms of certain strains may persist in the central nervous system although the other tissues have been sterilized (Buschke and Kroó, 1923 ; Kritschewski and Brussin, 1927 ; Prigge and Rothermundt, 1928). The infection with *Sp. muris* in mice has been extensively investigated because its susceptibility to chemotherapeutic agents shows a close parallelism with that of *Tr. pallidum* in man and susceptible animals.

Schlossberger (1928) has pointed out that if quantitative relationships between curative dose and body-weight were the same for different species of host as for the mouse, an average man would require for the cure of infection with the organism of rat-bite fever a dose of 22 gm. of neosalvarsan. Actually from 1 to 3 or 5 doses, each of 0.45 to 0.6 gm., have proved effective in man. This discrepancy in different species of hosts is of great importance ; the advantage of greater therapeutic effectiveness in larger animals tends to counteract the disadvantage that substances are generally more toxic for these than for smaller animals. Both properties may be functions of the metabolism, which is more active where the

body-weight is small and therefore the surface is larger in proportion, but no definite laws in regard to the action of chemotherapeutic substances in this respect have been established.

In connection with problems of dosage it is of interest that Voegtlin (1929) found that rabbits in which treatment was begun on the fifty-fifth day after inoculation with *Tr. pallidum*, were cured in practically the same proportion by a series of four doses at six-day intervals of arsphenamine, each of 6 mgm. per kgm., as were cured by the same total amount (24 mgm.) administered in a single dose. Two doses totalling half this amount given at six-day intervals failed to effect cure in the majority of animals. The dose of 6 mgm., it is to be noted, is less than the maximal human dose.

Clinical observations on syphilitics had established the unlikelihood of an individual developing for a second time the primary sore and other early manifestations of infection. It was then discovered that this so-called immunity was frequently associated with a positive Wassermann reaction of the blood; further, a similar refractory state has been observed in monkeys after infection with *Tr. pallidum* and the organs of such animals have been shown to be infective for normal monkeys. With the introduction of salvarsan treatment apparent reinfection in human cases ceased to be rare. Also, monkeys which had been inoculated with *Tr. pallidum* and then presumably sterilized by treatment (as judged by non-infectivity of the organs of other similarly treated animals) were found soon to be susceptible to reinfection (Neisser, 1911). Accordingly, it became generally accepted that, following infection with *Tr. pallidum*, insusceptibility to develop local evidence of reinfection was proof that the original infection still persisted. Conversely, susceptibility to reinfection was taken as evidence of complete sterilization of the original infection. In the light of these criteria Kolle (1924) investigated the response to treatment of rabbits infected with *Tr. pallidum*; the conclusion was reached that when treatment, even of the most energetic description, was begun at an interval of longer than 90 days after inoculation complete cure was, as a rule, no longer possible. This was regarded as evidence that the tissues had undergone some alteration, so that they ceased to co-operate effectively with the drug (salvarsan) in such a manner as to kill the spirochaetes.

Clinical observations, as well as the behaviour of the Wassermann reaction, had also led to the conclusion that in the late stages of the infection, cure is difficult or impossible. Warthin (1916, 1918, 1929), as a result of histological investigation and the demonstration of spirochaetes, has gone further and holds the extreme view that syphilis never becomes perfectly healed, since there are always active latent lesions in the aorta, heart or elsewhere; he regards this as equally the case after modern forms of treatment and after the use of mercury. Chesney with Kemp and Halley (1924-7), however, have interpreted the experimental results in rabbits differently; they believe that when treatment is begun at a long interval after inoculation there has been time for a marked immunity

reaction to occur. The failure to develop local manifestations on reinoculation under such conditions is, therefore, attributed to such immunity and is not considered as conclusive evidence that the original infection still persists. It is to be noted that the immunity is most marked toward the homologous strain. A definite decision between the two views appears impossible at present on account of the difficulties which attend the detection of spirochætes when these are present only in scanty numbers. But Uhlenhuth and Grossmann's (1927) observations lend strong support to Chesney's view; they found that in animals which had been treated late and whose popliteal glands thereafter became non-infective, re-inoculation, although it led to no local lesion, nevertheless caused the organs or lymph glands in a proportion of cases to produce infection when inoculated into further rabbits. Such immunity, however, is not accompanied by the presence in the serum of demonstrable spirochæticidal antibodies, as has been clearly shown by Uhlenhuth and Grossmann recently and also by older observations.

An important and much debated problem which arises especially in connection with *Tr. pallidum* infections is the possibility that treatment which is insufficient to destroy all the organisms, may actually determine the occurrence of lesions. It has been observed that in certain cases of syphilis treatment with small amounts of salvarsan early in the stage of generalized infection (i.e. secondary stage) was rapidly followed by relapse in the form of a serious lesion in the cranial nerves, the neuro-recurrence. This phenomenon occurs in cases which have presented the usual manifest primary and secondary lesions, and so differs from the form of neurosyphilis commonly observed where the skin manifestations have been so slight that specific treatment has not been applied.

According to Ehrlich, the neurorecurrence is due to renewed activity of spirochætes which have persisted in protected situations in the central nervous system, while the rest of the organisms in the body have been killed before there has been time for an effective immunity reaction to occur. The persisting spirochætes then give rise to lesions which cause severe effects owing to their anatomical situation. This view regarding the nature of these relapses receives its strongest support from the fact that their occurrence is prevented by more vigorous treatment. Another explanation which has been advanced to account for such manifestations is that certain strains of *Tr. pallidum* have a special neurotropic affinity. In support of this clinical observations have been cited tending to show that infections from particular sources give rise to nervous lesions in an unusually large proportion of cases. The evidence from animal experiments as to the existence of neurotropic strains is not conclusive, however.

Again, in rabbits, in which generalized lesions following inoculation with *Tr. pallidum* are inconspicuous, Brown and Pearce (1920) have shown that, among other procedures, the use of therapeutic agents capable of inducing resolution of the primary lesions without completely destroying

the infecting organisms may alter the character of the infection and lead to the production of marked generalized lesions. It is questionable how far these observations may apply to the infection in man.

The so-called Jarisch Herxheimer reaction consists in the intensification of rashes or their appearance and the aggravation of syphilitic lesions in general shortly after the commencement of treatment ; this has been attributed to stimulation of the organisms by the drug. Libération of endotoxins following sudden destruction of the spirochætes may also account for some of these phenomena, but the complete explanation is obscure. In order to avoid such 'shock reactions', which may be serious in their effects when the nervous or vascular systems are affected, it is often advantageous to commence treatment with drugs such as bismuth or mercury which act relatively slowly on the infection.

While the above-mentioned spirochæte infections are influenced by compounds of arsenic, antimony and bismuth, they differ from trypanosomiasis generally in responding very little to the purely organic compounds hitherto investigated. The only drug which so far has been found to exert a powerful influence on *Leptospira* infections is bismuth (Sazerac, Nakamura and Kitchevatz, 1927 ; Uhlenhuth and Seiffert, 1928).

Attempts at the artificial production of drug-resistant spirochætes, including *Tr. pallidum*, have met with little success (Margulies, 1910 ; Rothermundt and Dale, 1911) ; although Margulies and Gonder (1912<sup>1</sup>) succeeded in developing arsenic-resistant strains of *Sp. recurrentis* and *Sp. gallinarum* after very prolonged treatment. Gonder (1914) found that this acquired drug-resistance persisted on passing the spirochætes through ticks or directly from mouse to mouse. On the other hand, certain strains of *Sp. recurrentis* are naturally resistant to arsenicals *in vivo* (see Gray, 1929). In cases of recent syphilis which have resisted treatment with salvarsan the infection when transferred to rabbits responded to the drug in the usual way in the few cases tested (see Nothhaas, 1927 ; Oelze, 1928).

#### **The Mode of Action of Chemotherapeutic Agents in Infections with Trypanosomes and Spirochætes : Ehrlich's Receptor Theory.**

In order to provide a rational explanation of the phenomena of chemotherapeutic action and of relapses in infections due to trypanosomes and spirochætes, Ehrlich applied a modification of his receptor theory involving the conception of chemoceptors and nutriceptors. In the first place it is to be noted that many of the most effective therapeutic agents such as trypan-red, the arsenicals atoxyl and arsacetin (both containing pentavalent arsenic) and salvarsan, and also Bayer-205, have apparently very little effect on the vitality of the trypanosomes *in vitro*, at least as indicated by their motility. In the case of arsenicals it was found that certain compounds containing trivalent arsenic were actively trypanocidal

*in vitro*, e.g. arsenious acid and especially para-aminophenylarsenious oxide, which is produced by mild reduction of atoxyl. Therefore, it was supposed that such toxic derivatives are produced *in vivo*. Certain experimental evidence appeared to show that the host's tissues converted the drug into the actively toxic reduction product, since mixtures of minced tissue (liver, lung, muscle), even after boiling, along with atoxyl, on standing for a time acquired trypanocidal action *in vitro* (Levaditi and Yamanouchi, 1908).

The evidence that the therapeutic effect *in vivo* is actually due to such products resulting from the action of the host's tissues on the drug was, however, indirect. Thus, Ehrlich pointed out that individual mice varied considerably in their tolerance of atoxyl, which he ascribed to differences in their capacity for reducing it, the reduction product being highly toxic for higher animals as well as trypanosomes. Further, in highly tolerant animals the therapeutic effect of large doses of atoxyl on the trypanosomes was certainly not greater than that of smaller doses in less tolerant individuals (see Roehl, 1909<sup>1</sup>).

The rate at which the parasites disappear from the blood after an intravenous injection of different chemotherapeutic agents has also been adduced as evidence regarding the chemical changes which they may require to undergo before they affect the trypanosomes (Voegtlin and Smith, 1920). Thus, substances of the organic arsenious oxide type and trivalent antimony (antimony lactate) act rapidly, whereas after administration of salvarsan and the pentavalent arsenicals there is a latent period of some hours before the parasites begin to disappear. Voegtlin and Smith believe that during this time the latter drugs are being converted into the actively trypanocidal form. In the case of *Sp. recurrentis*, however, according to Feldt and Schott (1927) the arsenious oxide compound does not produce rapid lethal action either *in vitro* or *in vivo*.

Moldovan (1914) applied an ingenious method and obtained results which suggest strongly that the drug (atoxyl) combines directly with the trypanosomes and that lethal action follows, presumably as a result of the elaboration of reduction products in the body of the parasites. He showed that nagana trypanosomes suspended in salt solution underwent active multiplication for some hours when enclosed in collodion sacs which were placed in the peritoneal cavity of rats. But when a dose of atoxyl was injected intraperitoneally at the beginning of the experiment the parasites died and underwent solution. The sacs were impermeable to proteins, so that only dialysable substances could penetrate from the host's tissues. When the drug was administered subcutaneously the result was the same as when it was introduced intraperitoneally. When an atoxyl-fast strain of trypanosomes was used under conditions similar to those in the above experiment the parasites multiplied in spite of the presence of the drug. Experiments with the normal strain of trypanosomes in which salvarsan was injected subcutaneously gave the same result as with atoxyl. Also when collodion sacs containing *Sp. recurrentis* or

*Sp. gallinarum* were introduced into the peritoneal cavity of rats and fowls respectively, administration of salvarsan to the animals caused degeneration and solution of the organisms. Again, it has been shown in several instances that when the parasites are treated with a solution of the drug *in vitro*, e.g. trypanosomes with neosalvarsan (Castelli, 1913) or Bayer-205 (Reiner and Kőveskuty, 1927), and are then separated, they have been so modified that, although still actively motile, on inoculation into susceptible animals infection may not occur. Such experiments support strongly Ehrlich's conception that 'corpora non agunt nisi fixata'—combination of the drug with receptors (chemoceptors) in the protoplasm of the parasites is the essential preliminary to their destruction.

Apparently then in the instances considered the evidence points to a direct action of the drug on the parasites. Whether purely chemical factors are concerned, as Ehrlich's conception of chemoceptors implied, or whether physico-chemical influences are involved can scarcely be decided; in any case the effect of chemotherapeutic agents depends upon their chemical constitution. The action of trypaflavine on the blepharoplast of *Bodo* in cultures *in vitro* (p. 507) is a striking parallel to the effect of the drug on trypanosomes *in vivo*, and here again strong evidence is afforded of direct action. On the other hand, trypan-red, one of a series of trypanocidal compounds belonging to the group of bis-azo dyes, cannot be shown to affect the parasites *in vitro* (Ehrlich and Shiga, 1904). Blood containing *T. equinum* which has been in contact with a strong solution of trypan-red is still infective after the dye has been removed by centrifuging. In the body such dyes as well as producing trypanocidal action lead to vital staining of the reticulo-endothelial system and certain gland cells, but whereas the staining of the tissues persists for weeks or months the therapeutic effect disappears in several days as shown by prophylactic experiments. Accordingly the evidence for direct action is wanting. Trypan-red has been shown to abolish multiplication *in vitro* of *Paramecium*, and so to lead to death of the culture without affecting other properties of the organisms such as their motility (Busck, 1906); it has been supposed that a similar inhibition of the proliferative powers of trypanosomes may occur *in vivo*. Ehrlich's (1909<sup>3</sup>) experiment with a strain of *T. brucei* which was partially fast to arsenicals, also illustrates the difference between the functions of motility and of proliferation in trypanosomes. This strain was immobilized *in vitro* in three to seven minutes by 1 : 500 of an arsenical compound 'trypocid', whereas the original strain did not lose its motility until after 30 to 40 minutes under the same conditions. But when mixtures of parasites and varying dilutions of the drug were at once injected subcutaneously into mice a concentration of 1 : 2,000 along with the resistant strain led to infection, whereas the original strain failed to infect in the presence of 1 : 4,500.

The phenomena of drug-resistant strains formed a basis for the chemoceptor theory, the resistance being attributed to diminution in the affinity of the chemoceptors of the parasites for the drug. The fact that

living oxazine-resistant trypanosomes were not stained by the dye whereas the susceptible strain became stained is strong experimental evidence in support of direct action. As has been seen also, drug-fast strains may manifest their resistance *in vitro*, the arsenic-resistant trypanosomes remaining long motile in concentrations of the reduction product which rapidly immobilize the normal strain. In order to explain different grades of resistance to members of the same therapeutic group, as in the case of the four arsenic-fast strains, an elaboration of the theory was necessary and it was supposed not merely that the avidity of the chemoceptors might vary within wide limits, but also that the parasites contained several sorts of chemoceptors each of which anchored a different side chain of the drug. Again, the phenomena of therapeutic interference are compatible with the chemoceptor theory. That the host plays a definite part in the effect of such chemotherapeutic agents, however, would appear from the observation that a strain of trypanosomes may be resistant to a given drug in one species of host and not in another. It is clear that the effect of a chemotherapeutic agent will depend on the metabolism of the host as well as on that of the parasites. Excretion or destruction of the drug and also its fixation by the host's tissues will tend to occur and these processes vary in different species and even in different members of the same species. Accordingly, the irregularity of action of chemotherapeutic drugs in different species and individuals is not surprising; the most suitable substances are those which are most uniform in their reactions with the host. This was implied by Ehrlich when he emphasized the need for marked 'parasitotropic', as opposed to 'organotropic', properties in a good chemotherapeutic agent.

The development of relapse strains resistant to serum antibodies, especially in view of the possibility of producing this result rapidly *in vitro*, was explained by nutriceptors, which were conceived as essential for maintaining the nutrition of the parasites. When the antibody combined with these receptors they disappeared and the survival of the strain depended on the development of a new type of nutriceptor; the parasites which evolved constituted the relapse strain.

Both serum-resistant and drug-resistant strains are, according to Jollos (1924), of the nature of 'persisting modifications' which as a rule disappear in time. These characters being unrelated to structures connected with the sexual cycle of the parasites, are therefore not hereditarily transmitted and so ought not to be classed as mutations.

### Malaria.

The administration of quinine and certain other of the cinchona alkaloids has a marked action in causing disappearance from the blood of the three species of malarial parasites of man. At the same time the pathological effects of malarial infection, especially the febrile attacks, are suppressed. There is, however, a tendency for a proportion of the

parasites to persist and therefore relapses are common. Since relapse may not occur for a long period it appears most likely that the organisms are held in check by antibodies, but as regards the existence of these no direct evidence has been obtained (Schilling, 1927; Plehn, 1928). A number of problems arise owing to the organisms passing through a cycle comprising both intracorporeal and extracorporeal stages and also the formation of the gametocytes. While it has been held that the gametocytes are relatively resistant to treatment with quinine, differences in the vulnerability of the organism at the various stages of the asexual cycle have not been conclusively demonstrated. So far as is at present known, antimalarial properties are confined to a few substances comprising, in addition to quinine and its congeners, methylene blue (Ehrlich and Guttman, 1891) and salvarsan, and the recently introduced synthetic drug plasmoquine (Roehl, 1926<sup>a</sup>). Beyond the fact of their action in malaria there is no intimate relationship of chemical or other nature between these groups of drugs.

The investigation of substances for antimalarial properties has been greatly facilitated by the observations due to Kopanaris (1911), the Sergents (1921<sup>1</sup>) and others that similar parasites in birds show a resemblance to those of human malaria in their response to a chemotherapeutic agent such as quinine; and it was in this way that the properties of plasmoquine were discovered. The infections of birds, which are caused by several different species of parasites, are not readily influenced by quinine. It is possible, however, to determine in canaries the amount of the drug which when introduced once daily into the stomach for six days in succession will cause a delay of at least five to six days in the appearance of *Plasmodium præcox* in the circulation beyond the normal incubation period of four to five days, treatment being begun at the time of inoculation (Roehl, 1926<sup>a</sup>). By this method the minimum effective dose of quinine is one-quarter of the maximum tolerated dose; for plasmoquine the ratio is one-thirtieth. Treated birds were found at times to harbour small numbers of parasites, since their blood when injected into normal animals caused infection to develop in the latter (this latent infection supervenes regularly in untreated birds which survive the acute stage). Reinoculation of canaries harbouring such a latent infection did not cause parasites to reappear acutely in numbers in the blood; on the other hand, completely cured animals, whose blood was not infective for others, developed promptly a fresh infection after reinoculation.

The immunity to reinoculation of birds with latent infections does not depend on serum antibodies (Taliaferro, 1929). When treatment is delayed until the blood contains parasites the drug causes morphological changes in them and they may then temporarily disappear; but relapse always follows and a chronic infection is established. According to Kopanaris (1911) the administration of salvarsan in bird malaria aggravates the infection, while Marks (1914) has recorded that the infection is greatly

influenced by feeding the birds with methylene blue. Collier and Krause (1929) have found that the infection of rice finches with *Halteridium* (*Hæmoproteus*) forms a suitable object for demonstrating the therapeutic action of plasmoquine.

As regards the mode of action of plasmoquine on bird malaria, there is the same difficulty as in the case of quinine in the human subject. Thus, a dose of plasmoquine which has a marked effect in delaying the appearance of parasites in the blood when administered to birds some hours after inoculation, is without action when given as short a time as two hours beforehand. Similarly in man, inoculation with plasmodia succeeds three hours after the last of a long series of 1 gm. doses of quinine taken by mouth on alternate days (Roehl, 1926<sup>3</sup>). In human cases inoculated with blood containing simple tertian parasites the administration of daily doses of quinine for some days afterwards has prevented infection. But this contrasts with the result after inoculation by the bites of infected mosquitoes, where such prophylactic treatment is without effect (Yorke and Macfie, 1924). Mühlens and Kirschbaum (1924) have shown that a mixture of equal parts of defibrinated blood containing simple tertian parasites along with 1:5,000 quinine solution was still infective on inoculation after incubation for 12 hours at 37° C. Yorke (1925) with Macfie failed also to kill the parasites by incubating them along with mixtures of quinine and extracts of fresh liver or muscle. Thus, the evidence for direct action of antimalarial drugs on the parasites is so far not conclusive, although many facts have been interpreted in this sense (Giemsa, 1927). Bass (1922) found that quinine dihydrochloride in a concentration of 1:2,700 in a blood culture containing *Plasmodium falciparum* prevented growth and finally killed the parasites, even when the fluid was removed by centrifuging after 20 minutes and was replaced by serum from the same source. It may be, as Roehl suggests, that the combination between the protoplasm of the parasites and the drug is highly reversible, or, as is indicated by the necessity for prolonged treatment in cases of malaria, that the action of the drug on the organisms is exerted only very slowly.

Morgenroth (1918) and his co-workers demonstrated that quinine and optoquine are fixed *in vitro* by red corpuscles and again readily given off. One method by which this was shown in the case of optoquine consisted in adding a solution of the alkaloid dissolved in normal saline to a suspension of red blood corpuscles from man or animals, then centrifuging and applying the sediment and the supernatant fluid respectively to the rabbit's cornea; that on which the sediment was placed became anaesthetized, thus showing the action of the alkaloid, whereas the fluid was without effect. The view was then developed that the red corpuscles, having concentrated the alkaloid, exert a negative chemotaxis towards the parasites, which when unable to penetrate the red cells are soon destroyed. This 'repulsion theory' has not been generally accepted, since 95 per cent. of a dose of quinine injected intravenously disappears

almost at once from the circulation (Rona and Bloch, 1921), although traces continue to be present for many hours (Hatcher and Weiss, 1926). The walls of the capillaries apparently fix the drug, as one-quarter of the dose which has been injected intravenously can be recovered by perfusion from an animal exsanguinated a few minutes after the injection ; from this source a depot of quinine may be maintained by the organs, small amounts continually passing into the blood-serum for an extended period. It is of interest, however, that Hegner, Shaw and Manwell (1928) found that of a small number of compounds belonging to the quinine series those which were not selectively taken up by the red corpuscles were without therapeutic action on bird malaria, whereas the active substances were taken up.

Attention has been repeatedly drawn to the relative ease with which cure is effected in simple tertian malaria transmitted by the injection of infected blood as compared with that naturally acquired ; it is generally held that this is due to treatment in the former case being undertaken at an early period of the infection. The parasites do not appear, however, to become quinine-fast, since infections derived from patients who had resisted treatment, showed the normal response to quinine in other subjects (Mühlens, Weygandt and Kirschbaum, 1920). A similar result has been got in bird malaria by the Sergents (1921<sup>2</sup>).

The effect of plasmoquine in causing disappearance of crescents from the blood in cases of the tropical infection (Roehl, 1927) is specially noteworthy, as these forms are particularly resistant to treatment with quinine (see Mühlens, 1929). Apparently the earliest result of the action of plasmoquine on the crescents is to render them non-infective for mosquitoes (Barber, Komp and Newman, 1929).

#### Piroplasmosis.

Nuttall and Hadwen (1909) first showed that in the serious disease of dogs due to a *Piroplasma* the injection of a dose of trypan-blue (or trypan-red) caused the parasites to disappear from the peripheral blood. This is the only drug which acts with certainty. Following the administration of the dye the organisms showed morphological changes, especially in the extracorporeal forms. Reappearance of parasites in the blood tended to occur after 9 to 12 days ; but, unlike the original attack in untreated animals, the relapse as a rule was not accompanied by illness. Similar results were obtained in red water of cattle [infection with *Piroplasma (Babesia) bigemina*] and also in other animals infected with *Babesia* parasites. But East Coast fever (infection with *Theileria parva*) failed to respond to treatment. These results have been generally confirmed. Bayer-205 is without action on piroplasmosis of dogs (Roehl, 1926<sup>3</sup>).

**Amœbic Dysentery.**

The curative action of the ipecacuanha alkaloids, emetine and cephæline, on amœbic dysentery was elucidated by Dobell and Laidlaw (1926) (see also Laidlaw, Dobell and Bishop, 1928), who showed that these drugs are directly and specifically toxic for *Entamœba histolytica* in actively growing cultures. Thus, they are at least 50 times more toxic for *E. histolytica* than the other ipecacuanha alkaloids tested, and they are similarly more active than quinine. Further, *E. coli* tolerates a concentration of emetine which is 100 times that lethal for *E. histolytica*. The killing action is exerted very slowly even by relatively high concentrations, 1 : 1,000 of the drug taking many hours to kill *E. histolytica*; but high dilutions (1 : 50,000) prevent regular and continuous growth in cultures. In a suitable liquid medium consisting of serum diluted with Ringer's solution to which a trace of rice starch is added, and provided the reaction is not too acid, emetine in a dilution of 1 : 500,000 is lethal for *E. histolytica* in four days. Apparently it is not possible to develop emetine-resistant races of *E. histolytica*. That the host itself plays an important part in conducting to cure is, however, indicated by the fact that infection with the same strain of *E. histolytica* as was curable in man, could not be cured in kittens (Dale and Dobell, 1917). Dobell and Laidlaw consider that this difference may be explained by the modes of excretion of emetine being different in man and the cat—the alkaloid passing into the intestine in the former, whereas in the latter animal it is excreted mainly in the urine.

**Bacterial Infections.**

Attempts at chemotherapeutic treatment of infections with the ordinary bacteria such as pneumococci, streptococci and tubercle bacilli have developed along two main lines, (a) the employment of antiseptic substances whose antibacterial properties are relatively high as compared with their toxicity for mammalian tissues, and (b) the metal-salt therapy of Walburn, the substances being used in doses which have probably no direct action on the organisms. Details are given in the articles dealing with the various organisms; here the general principles will be discussed.

**Antiseptics.**

The longest known antiseptics such as mercuric chloride, phenol and its derivatives, and the chlorine group are all highly toxic for the tissues of higher animals as well as for bacteria; they are, therefore, classed as protoplasmic poisons. Evidence of this general affinity for proteins is shown in the case of the most active members of these classes by the great diminution in antiseptic effect which occurs when they are mixed with an albuminous fluid such as blood-serum; and this applies to such extremely potent compounds as tetrachlor-orthobiphenol introduced by Bechhold and Ehrlich (1906). Many basic organic dye-stuffs are very highly antiseptic (Behring, 1889; Koch, 1890). In general their lethal

action on the organisms develops relatively slowly ; short of killing the bacteria they inhibit multiplication—bacteriostatic action. Since concentrations which are powerfully bacteriostatic can be brought into contact with mammalian tissues without causing serious damage to the latter, it appeared that such properties might be utilized with advantage in the treatment of infections. Among the substances of this kind to which attention was drawn were the triaminotriphenylmethane dyes, gentian violet and its analogue ethyl violet (Stilling, 1891). Later investigations have shown that these properties are exhibited to a greater or less extent by several classes of basic dye-stuffs, including also the diaminotriphenylmethane compounds (malachite green and brilliant green), aminoacridine derivatives (acriflavine or trypaflavine, proflavine and rivanol), aminoanilquinoline dyes and certain organic compounds of mercury, e.g. mercurochrome-220 (see Vol. I, p. 202). Selective action is strikingly shown by many of these basic antiseptics. The work of Churchman especially established that gentian violet (or crystal violet) is much more powerfully bacteriostatic toward Gram-positive organisms such as staphylococci, diphtheroids and aerobic sporing bacilli than toward a Gram-negative organism like *B. coli* ; this relationship is not invariable, however. As an extreme example of selective action, Browning, Cohen and Gulbransen (1922) found that the carbocyanine dye sensitol red was about 2,000 times as powerful an antiseptic for *Staphylococcus aureus* as for *B. coli* in a medium consisting of dilute peptone water. On the other hand, many of the aminoacridine compounds, e.g. acriflavine, and the anilquinolines are as active toward *B. coli* as toward staphylococci (Browning, Cohen, Ellingworth, Gaunt and Gulbransen, 1922, 1926, 1928). Many of the organic compounds, like the inorganic antiseptics, are inhibited in their action by the presence of serum ; a number of representatives of the aminoacridine and anilquinoline series, however, are not affected in this way by serum. As regards the slowness with which most of these basic substances exert their killing action on organisms, Koch (1890) early realized that a therapeutic agent need not be actually lethal for the causal organisms *in vivo*. It is clear that if organisms are inhibited the antibacterial mechanisms of the host, especially the phagocytes, will then be enabled to come into play.

*General infections.* In a general infection it is of course necessary that the antiseptic should become diffused. Morgenroth and his co-workers discovered that ethylhydrocupreine (optoquine), a substance closely related to quinine, when injected subcutaneously cured mice infected with pneumococci even at an advanced stage when the organisms were present in the blood (see Vol. II, p. 225). It has not been possible to reproduce this result in other animals or in infections with other organisms. Thus, acriflavine or proflavine, in spite of their very powerful bacteriostatic and antiseptic properties, failed to cure staphylococcus septicæmia in rabbits (Tubby, Ferguson, Mackie and Hirst, 1919), although the blood-serum can be rendered definitely bactericidal for staphylococci and other

organisms for a short time after an intravenous injection of a dose of the dye which has no harmful effect on the animal (Browning and Gulbransen, 1918). Schiemann (1923), however, succeeded in saving the life of a proportion of mice inoculated intraperitoneally with an atypical strain of pneumococci by administering a subcutaneous injection of a solution of trypaflavine (acriflavine) five minutes later. On the analogy of drug-fast trypanosomes, virulent strains of pneumococci resistant to optoquine have been developed (Morgenroth and Kaufmann, 1912; Lewy, 1925).

*Local infections.* When the chemotherapeutic agent can be brought into close contact with the organisms in a localized infection circumstances are much more favourable for effective action than when the organisms are widely distributed throughout the body. Lister (1867<sup>1&2</sup>, 1909) originally dealt with such conditions when he applied carbolic acid in the liquid state or, later, as a watery solution to the infected tissues exposed in compound fractures. Afterwards, Stilling (1891) and others suggested that the antiseptic properties of methyl violet, ethyl violet, &c., for staphylococci and other organisms might be utilized in the treatment of infections on surfaces such as the conjunctiva or in joints.

The difficulty of producing localized infections in small animals has interfered considerably with the experimental investigation of this subject. In certain cases, however, suitable test infections have been found. Thus, virulent *C. diphtheriae* introduced into recent skin wounds in guinea-pigs remain localized, death being due to general intoxication. The action of therapeutic agents can be determined by their capacity to cause survival of the animals when the wounds are treated at an interval after inoculation (Feiler, 1920; Braun and Goldschmidt, 1927). It was shown in this way by Browning and Gulbransen (1925) that swabbing of the infected wounds once for 75 seconds with 1 c.cm. of antiseptic solution at a period up to two hours after inoculation led to survival of the animals when acriflavine in dilutions of 1 : 100 to 1 : 1,000 was used; this result is due to killing of the organisms and not to destruction of their toxins (Braun, 1922). Similar treatment of the wounds with 0·85 per cent. or 5 per cent. NaCl solution did not prevent the acutely fatal result. In comparative experiments with acriflavine and carbolic acid solutions the ratio of therapeutic effectiveness exceeded 50 : 1. Cure of otherwise fatal infections has been obtained in experiments on similar lines carried out with streptococci in mice (see Vol. II, p. 142; Collier and Bernhagen, 1928).

Morgenroth, along with Abraham and other co-workers in investigations on the chemotherapy of local streptococcal infections, injected streptococci into the subcutaneous tissue in mice and then immediately followed this by an injection of a solution of the therapeutic agent into the same area. The effect was judged by killing the animals after 24 hours and making cultures from the site of inoculation. The method has been claimed to yield very uniform results and to be of value for comparing the therapeutic action of different substances. It has been found, however, that the

varying susceptibility of individual animals tends to interfere with the constancy of such tests (Browning and Gulbransen, 1928<sup>1</sup>; Collier, 1929).

Obviously the therapeutic utility of a locally acting antiseptic may depend on factors additional to its bactericidal property as measured *in vitro*. In order to be effective, substances should be relatively little toxic for the tissues locally, so that interference with the natural defensive mechanisms may not result. Attempts have been made to assess this property *in vitro* by observing the effect of the drug on the phagocytic action of leucocytes (see Browning, Gulbransen, Kennaway and Thornton, 1917), or on their bactericidal activities (Fleming, 1924), or on the growth of tissue cultures (Mueller, 1919). Especially in the latter case it must be remembered, however, that the conditions are highly artificial; when cells are kept outside the body and are removed from the naturally renewed source of nutrition provided by the capillaries it is unlikely that they possess the vitality shown under normal circumstances. Thus, in granulating wounds treated with 1 : 1,000 solutions of the acridine antiseptics (acriflavine or proflavine) mitoses have been found in various types of cells—endothelial, epithelial, large mononuclears and fibroblasts—at an average depth of 0·11 mm. below the surface (Blacklock, 1929).

A further point of importance is that in the case of absorption of the drug there should be no poisonous action on any vital organ. A factor which may also interfere with therapeutic action is the capacity of fluid or solid constituents of the tissues to inactivate the antiseptic. Perchloride of mercury and chloramine-T are examples of powerful antiseptics which suffer great reduction in bactericidal power when added to a protein medium such as serum. As has been mentioned, there are, however, many powerfully antiseptic dyes such as acriflavine and proflavine and members of the anilquinoline series which are not affected by the presence of serum. Solid constituents of the tissues also may fix the antiseptic and this is to some extent the case with practically all such substances. The consequences of this combination may be either to inactivate the antiseptic, or, on the other hand, to hold it in a 'depot' and thereby to secure the advantage of prolonged action on the organisms. The latter explanation was suggested by Morgenroth (1919) in order to account for the fact that substances which were similar in their antiseptic action *in vitro* might show marked differences when tested on the same organisms locally *in vivo*, e.g. against staphylococci in the subcutaneous tissues of the mouse (Morgenroth and Wreschner, 1923).

A useful method for ascertaining the action on local pyogenic conditions in the human subject is to apply different therapeutic agents to several similar infected areas such as burns of equal severity and in similar situations in one individual; also the substances may be applied in alternation to one area. By following this procedure, Graham (1925) showed that under the conditions observed acriflavine, 1 : 1,000, was effective in controlling suppuration, whereas a saturated solution of boric acid was not.

So far no antiseptic is known which influences the intestinal flora.

*Metal-Salt Therapy.*

Walbум (1921) demonstrated that the injection of certain metal-salts rapidly caused a temporary increase in the antibody content of the blood-serum in actively immunized animals (Walbум and Mörch, 1923). Although a general confirmation of his results is lacking, it appears that this is due to inability to control all the factors concerned (see Mackie, 1925). Proceeding from this observation, Walbум investigated the effect of injections of solutions of metallic salts on infections with various organisms and also on the action of bacterial toxins (1925, 1926, 1927, 1928, 1929<sup>1 & 2</sup>). He found that animals could be protected against lethal doses of organisms or toxins in many cases by a series of subcutaneous or intravenous injections of certain metal-salts. Möllgaard (1924) had already drawn attention to the curative action of a gold sodium thiosulphate compound (sanocrysin) in animals infected with tubercle bacilli, which, however, he apparently attributed to the direct action of the drug on the organisms. The effect of this compound was systematically investigated by Madsen and Mörch (1926, 1928), who confirmed and extended Möllgaard's results. Certain striking facts appeared from the very extensive series of experiments carried out by Walbум and by the latter workers, namely, that (1) the same metals are not effective against all the pathogenic agents, also the same metal may not act similarly in animals of different species ; (2) there is an optimum dosage of the salt ; (3) the time of commencement of treatment is important ; (4) the virulence of the infecting organisms plays a part, since in certain circumstances an infection with highly virulent organisms may be more amenable to treatment than one with the same organisms but of lower virulence ; (5) the diet of the treated animals may have an important influence in determining the results of treatment.

*Specific action of metals depending on the pathogenic agent and on the species of host.* In the case of mice infected with the 'ratin' strain of organisms belonging to the *Salmonella* group, the chlorides of caesium and iridium alone were curative out of a large series of metals tested. Cadmium chloride, on the other hand, led to the cure of tuberculosis in mice. Manganese chloride when administered to mice inoculated with virulent tetanus spores first rendered the latter non-virulent and finally led to their death ; this salt also protected mice against the endotoxins of staphylococci and dysentery bacilli, while in tuberculous guinea-pigs treated with it a dose of tuberculin did not produce shock. The effect which may be exercised by the species of the host appears from the fact that rabbits infected with virulent tubercle bacilli were cured by injections of cadmium chloride, whereas such treatment in infected guinea-pigs frequently had merely the effect of altering the distribution of the lesions, but the animals died of the disease.

*Dosage.* Walbум found practically universally that there was an optimum range of dosage and that an increase beyond this failed to

influence the various infections and intoxications or even accelerated death, although the amounts which led to these harmful effects were not in themselves toxic for the host.

*Time of commencement of treatment.* In certain cases the metal-salt exerted its effect when administered either before or immediately after inoculation. Thus, mice inoculated with 'ratin' bacilli were cured when treatment with caesium chloride was begun as late as 2 days after inoculation. Also protection was conferred, as shown by the survival of animals inoculated 2 to 9 days after the last of a series of prophylactic injections of caesium chloride; and similar results were got after feeding with this salt. On the other hand, in Madsen and Mörch's experiments on the treatment of tuberculosis in rabbits with sanocrysin, cure resulted in 24 out of 30 animals when administration of the drug was begun at an interval of 5 to 12 days after inoculation with highly virulent tubercle bacilli. But when treatment was begun at the time of inoculation the fatal course of the infection was practically uninfluenced.

*Virulence of the infecting organisms.* In animals infected with tubercle bacilli of high and low virulence respectively, it was found by Madsen and Mörch that the former tolerated sanocrysin well whereas the latter tended to develop shock after its administration. Accordingly, for the treatment of infections with organisms of low virulence it was necessary to use smaller doses of the drug, and it was advantageous also to inject homologous antituberculous-serum. With certain tubercle bacilli of lower virulence sanocrysin failed to effect cure (Bang, Madsen and Mörch, 1928). According to Walbum, manganese and cadmium are the only metals capable of curing rabbits infected with highly virulent tubercle bacilli when treatment is begun at an advanced stage of the infection.

*The influence of diet.* Walbum (1929<sup>2</sup>) demonstrated that in mice infected *per os* with 'ratin' bacilli the success of the treatment by injections of caesium chloride depended on the diet of the animals. When they were fed on wheaten bran and bread moistened with water, cure resulted, but when the bread was moistened with milk, fatal infection developed. The effect of the therapeutic agent appeared also to be favoured by withholding food overnight before inoculation and treatment.

*Combined therapy.* It was shown also that metal-salts which were therapeutically inactive alone might be effective when administered along with homologous vaccine or with antiserum. For instance, neither the injection of manganese-salt by itself nor of a vaccine of killed 'ratin' bacilli alone cured mice infected with these organisms, but the combination of the two agents effected cure. Similarly, combined treatment with the manganese-salt and an otherwise insufficient dose of antiserum cured mice which had received a dose of tetanus, diphtheria or dysentery toxins fatal for untreated controls. Here also, to judge by the results in treatment of tetanus intoxication, the addition of milk to the diet interfered with the curative action of the metal-salt.

### General Considerations.

As has been seen, it is possible by the administration of substances with defined and often relatively simple constitution to cut short infections which otherwise would prove acutely fatal or would pursue a chronic or relapsing course. Such results may be obtained in a variety of infections, protozoal, spirochaetal and bacterial ; and complete sterilization of the causal organisms has been effected both in local and in general infections. In conditions such as trypanosomiasis of small animals, compounds of widely different chemical constitution have proved effective, but in others, for instance, pneumococcus septicæmia in mice, only one active substance has been found so far.

When the parasites are killed under the influence of the drug conditions are suitable for their acting as antigens. In certain cases it is easy to immunize normal animals with a vaccine of the killed organisms, e.g. with *Spirochæta gallinarum* ; hence the chemotherapeutic agent by leading to the death of the spirochaetes sets the immunity mechanism in operation. The serum of the treated animals has then pronounced antibody properties and exerts prophylactic and curative action. In trypanosomiasis there is a similar result, but as a rule the immunity which is produced is neither of high intensity nor long duration. The evidence is conclusive, however, that the process of sterilization initiated by the chemotherapeutic agent may be completed by the immunity reaction. The question remains as to whether the chemotherapeutic agent conduces in some special manner to the immunity response. It is, of course, well established that in a number of infections the presence in the body of living organisms is essential for the development of effective immunity. Accordingly, the therapeutic agent in these instances most probably favours immunization mainly by controlling the activity of the parasites, which remain alive sufficiently long to stimulate an immunity response which dead parasites are incapable of inciting ; this is well illustrated in trypanosomiasis.

Where complete destruction of the parasites is not achieved, relapse tends to occur and the results of such a relapse vary. The course of the infection may then progress much as in an untreated animal ; this is the rule with trypanosomiasis of small rodents. Again, the infection may be profoundly altered and the host may remain free from symptoms although harbouring living organisms. This result may depend on modification of the parasites ; thus, it has been found that when a trypanosome infection in the mouse has been rendered chronic as a result of treatment, the parasites on transference to another animal proved to be of diminished virulence. An immunity reaction on the part of the host may also account for the mitigation of the infection following non-sterilizing treatment. This is illustrated in piroplasmosis ; on transferring the blood from the immune but chronically infected animal to a susceptible one, an attack of the disease is liable to result. The partial destruction of the parasites,

therefore, may be associated with a greater or less degree of antibody formation. *Tr. pallidum*, on the other hand, appears to produce immunity of the tissues in the sense that in a chronically infected animal the organisms on reinoculation commonly fail to give rise to local lesions, but spirillicidal antibodies cannot be demonstrated in the serum.

The degree of specificity of therapeutic effects is a striking feature, which is apparent even in infections due to closely allied organisms. Trypanosomiasis in mice due to *T. brucei* is cured by less than one hundredth of the tolerated dose of Bayer-205, while that caused by *T. congolense* is influenced only by the highest doses ; on the other hand, natural infections with *T. congolense* are readily cured by antimony potassium tartrate (see Richardson, 1928). Even different strains of the same species of parasite may differ in their susceptibility to drug treatment and such alterations may take place apparently spontaneously. Salvarsan acts on both trypanosome and spirochæte infections, while sulphoxylsalvarsan is effective only against the latter. Quinine and plasmoquine, which influence infections with the malarial parasites both of man and birds, have no marked action on other protozoal infections. *Babesia* parasites, which also infest the red corpuscles, are destroyed by trypan-blue, which has no action in malaria. Optoquine cures pneumococcus septicæmia in mice, but fails in streptococcus infection. Again, in Walbum's metallic salt therapy, practically each bacterial infection is best influenced by a different metal.

The effects of therapy often depend on the stage of the infection at which treatment is begun. In acutely fatal infections which are septicæmic in their course or where toxic effects cause death, it is obvious that the tissues of the host may rapidly undergo serious damage, so that cure is most readily effected by early treatment. But where the disease is of longer duration other factors may operate. In bird malaria once infection is established the difficulty of effecting cure is greatly enhanced. In *Tr. pallidum* infections the changes occurring in the tissues after a time have been held to render sterilization impossible. On the other hand, in the treatment of tuberculosis in rabbits by sanocrysin it was found to be essential for cure to delay the commencement of treatment until some days after inoculation.

An important problem, knowledge of which is as yet deficient, concerns the comparative efficacy of a given drug in animals of different species. In general, small animals in proportion to their weight tolerate a bigger dose than large ones ; but against this may be set the rapid metabolism of the former, and therefore the probability that the drug will be sooner inactivated or excreted. Accordingly, it may be expected that, other circumstances being the same, in small animals conditions will be most favourable for securing brief action of a relatively high concentration of the substance, but that in larger animals the parasites will be subjected to its action for a more prolonged period although at a lower concentration.

A difficulty which attends such investigations is that few infections pursue a similar course as regards duration and type of lesions produced in many animals of different species.

The effect of variations in the chemical constitution of drugs on their chemotherapeutic action cannot be gone into here. But it should be remembered that alterations in chemical structure which affect the distribution of the drug in the body or the channel by which it is excreted may be of the greatest importance, since such properties may result in its reaching special situations in relatively high concentration. Thus, the good effects reported from the use of tryparsamide in sleeping sickness and in neurosyphilis may be due to this compound penetrating to the central nervous system, and so reaching the parasites situated there. A factor such as the hydrogen-ion concentration locally may affect chemotherapeutic action markedly. Acriflavine is excreted largely through the kidney, but its bactericidal action is very markedly reduced by a degree of acidity such as is common in the urine (Browning, Gulbransen and Kennaway, 1919); therefore, it is essential to render the urine as alkaline as possible in order to secure its full effect in infections of the urinary tract.

Chemotherapy may fail owing to the capacity of the parasites to undergo biological accommodation, which is apparent in the phenomena of acquired drug resistance and serum (i.e. antibody) resistance; both of these alterations are exhibited in striking degree by some species of trypanosomes. It is of interest that among bacteria the pneumococcus also readily develops drug-resistance. In the case of spirochaetes drug-resistance is almost negligible, but serum-resistance is readily developed by certain organisms belonging to this group; and the mechanism of relapses in relapsing fever is explained in this way. Drug-resistance is specific in the sense that substances can be arranged in classes, the development of resistance to one member of which involves resistance to the other members. But a strain resistant to one is normal in its behaviour towards the members of other classes; the classes may be chemically homogeneous, e.g. arsenicals are all characterized by their metallic constituent. On the other hand, trypaflavine which contains no inorganic component, also belongs to the arsenical class in this respect. The host, the parasites and the chemical constitution of the drug are all three operative in determining the development of drug-resistance; but this is by no means an invariable result of prolonged non-sterilizing treatment. Once such resistance has been acquired, however, it may be tenaciously retained when the parasites are passed through untreated animals. There are no certain methods of abolishing drug-resistance, although treatment with agents of a different type has been stated to do so and passage of the parasites through an invertebrate host may also lead to this result.

The phenomena of drug-resistance afford the theoretical basis for combined therapy, since two or more drugs of varying types which attack the parasite at different points may be expected to enhance each other's action. It is necessary, however, to guard against the possibility of

chemotherapeutic interference, which may result from unsuitable adjustment of dosage or intervals between the administration of the respective drugs. Another form of combined treatment which has been employed with success consists in the use of the chemotherapeutic agent together with an antiserum.

As regards the means by which chemotherapeutic agents bring about their effects, a powerful direct harmful action of the drug on the parasites is demonstrable in certain cases, and there is therefore good reason for concluding that this explains the curative property. In other instances, while the drug itself is comparatively inactive, reduction or oxidation products are toxic for the organisms and these presumably are formed by the metabolism of the host or the parasite and then act on the latter. Ehrlich has developed the conception of direct action in his chemoceptor theory, according to which one or more chemical groupings in the protoplasm of the parasites have a special affinity for chemical groups in the drug and thereby fix the latter. Many experimental facts have been discovered which support this hypothesis. On these lines in connection with the acid substances belonging to the type of Bayer-205, Roehl (1926<sup>1</sup>) has supposed that fixation is effected by combination with basic constituents of the cells. It must be noted, however, that the complexity of the phenomena as observed in drug-resistance and chemotherapeutic interference can scarcely be explained by the existence of any simple chemical structures corresponding to receptors.

The host probably seldom plays a purely passive role in the therapeutic process. In the first place, the vulnerability of its tissues determines the dosage and, therefore, the concentration in which the drug may come into contact with the parasites. Further, in the same species individual differences in the rate of excretion and other metabolic factors will directly affect the existence of the chemical substance in the body. Thus, the well-established variability in the effects produced by a constant dose of a drug in a series of similarly infected animals finds a ready explanation. It should also be recalled in this connection that the administration of an excessive dose which is poisonous to the host and so depresses its powers of defence, frequently exerts a 'contrary effect' and the infection, instead of responding to treatment, as would be the result with a smaller dose, is uninfluenced in its course. The part which the host plays appears, however, to be a preponderating one where, as in Walbum's experiments, a salt of caesium leads to the cure of a *Salmonella* infection in mice. Here both the nature of the drug and also the minute doses required seem to exclude any directly harmful action on the parasites. Further, Örskov and Schmidt (1928) demonstrated that under the conditions which they observed, treatment of animals infected by feeding with the organisms did not prevent their penetrating the tissues and producing after several days a bacteraemia as in the untreated controls. But the effects of treatment became apparent after these events, the tissues rapidly becoming sterile, whereas in the untreated controls progressive multiplication of the

organisms and death ensued. Walbum has suggested that the results are due to the metallic salt acting as a catalyst and so intensifying the defensive mechanisms of the tissues. A difficulty in accepting this explanation is the specificity of the effects; why should the ferments which stimulate the tissues to act on *Salmonella* organisms be activated by caesium, whereas the destruction of tubercle bacilli is brought about by means of cadmium or manganese, while tetanus toxin is decomposed under the influence of manganese?

Other evidence in support of the indirect action of chemotherapeutic drugs is the stimulating effect on antibody formation exerted by certain of these substances, and secondly the influence of interference with the reticulo-endothelial system. As regards the former, Agazzi (1909), Boehncke (1913) and others showed that the administration of various arsenicals, including salvarsan, intensified most forms of antibody production in response to the usual antigens in a variety of animals. The part played by the reticulo-endothelial system in modifying chemotherapeutic action has been the subject of much investigation. Two methods of experiment have been employed, namely, removal of the spleen and the so-called blockade of the system by injecting vital dyes or finely particulate matter such as carbon or iron oxide, which are taken up by the phagocytic cells. Frequently these procedures have been used in combination. Kritschewski (1927), with Meersohn (1926), Jungeblut (1927) and Feldt and Schott (1927) have concluded that splenectomy, especially when combined with blockade, reduces the effectiveness of chemotherapeutic agents in infections in mice due to *Sp. recurrentis*, *T. brucei* and streptococci. Similar results have been got in trypanosomiasis of rats after splenectomy (Lisgunova, 1928). It is obvious that where the procedures designed to affect the reticulo-endothelial system are by themselves sufficient to aggravate the infection, no conclusion can be drawn from the failure of therapy. But even where splenectomy and blockade had no effect of this kind the efficacy of treatment was generally reduced. This result was obtained in trypanosomiasis with a variety of drugs, including trypan-red, Bayer-205, pyronin, tryparosan, trypaflavine, atoxyl, neosalvarsan and tartrate of antimony; also the effect was apparent in treatment applied prophylactically as well as at a later stage of the infection (Rubinstein, 1928). Accordingly some fundamental mechanism would appear to be affected.

Several explanations have been advanced; thus the removal in the treated animals of cells which have a very marked storage capacity for many substances, including at least certain of the chemotherapeutic agents, has been supposed to prevent the formation of a depot of the injected drug necessary for its sustained action (see Kritschewski and his co-workers, 1929; Schlossberger, 1929). As evidence in support of this Kritschewski (1928) has stated that the addition of agar to a solution of tryparosan or Bayer-205 which is injected subcutaneously, compensates to some extent for the removal of the spleen by retarding excretion of

the drug. Again, in the case of those drugs which are not themselves highly parasiticidal, the transformation into the active form has been thought to occur within the phagocytes. Further, according to some observers, antibody production in general suffers impairment in animals whose reticulo-endothelial system has been reduced, and consequently the immunity response to the parasites damaged by the drug is interfered with. That the latter explanations can only be partial, appears from further experimental observations. For instance, the fact that the therapeutic action of the arsenious oxide compounds is interfered with as well as that of salvarsan, contradicts the view that failure of the mechanism of transformation of the latter into the former is responsible for the diminished therapeutic effect (Feldt with Schott, 1927, and Eisenmenger, 1928). Also, the failure of prophylactic action in the splenectomized and blockaded animals, as well as the fact that rapidly acting drugs, such as the arsenious oxide type, are reduced in their effect, is against interference with the immunity mechanism being the essential factor. It would appear that little regard has been paid to the summation of harmful influences in the animal involved in operative procedure along with the blockading injections (Kikuth and Regendanz, 1929)—the substances used for the latter purpose being practically always in doses which border on the toxic limit. A further point which must be borne in mind is that owing to the rapid power of renewal of the cells, complete or prolonged reduction of the functions of the reticulo-endothelial system is impossible (Cappell, 1929, 1930). Accordingly the part played by that important group of cells known as the reticulo-endothelial system in conducting to chemotherapeutic effects is still obscure.

## REFERENCES.

AGAZZI, B., 1909, *Z. ImmunForsch.*, Tl. I, Orig., 1, 736.  
 BALABAN, I. E. & KING, H., 1925, *J. Chem. Soc., Lond.*, **127**, 2701; 1927, *ibid.*, **2**, 3068.  
 BANG, O., MADSEN, TH. & MÖRCH, J. R., 1928, *Z. Hyg. InfektKr.*, **109**, 243.  
 BARBER, M. A., KOMP, W. H. W. & NEWMAN, B. M., 1929, *U.S. Pub. Health Rep.*, **44**, 1409.  
 BASS, C. C., 1922, *Amer. J. Trop. Med.*, **2**, 289.  
 BAUER, H. & BECKER, J., 1928, *Arb. Inst. exp. Ther., Frankfurt*, H. **21**, 10.  
 BECHHOLD, H. & EHRLICH, P., 1906, *Hoppe-Seyl. Z.*, **47**, 173.  
 BEHRING, E., 1889, *Z. Hyg. InfektKr.*, **7**, 171.  
 BLACKLOCK, J. W. S., 1929, *Brit. J. Surg.*, **16**, 401.  
 BOEHNCKE, K. E., 1913, *Z. Chemoth.*, **1**, 136.  
 BRAUN, H., 1922, *Klin. Wschr.*, **1**, 761.  
 BRAUN, H. & GOLDSCHMIDT, R., 1927, *Die Methoden d. tierexperimentellen Wundinfektion*, Berlin.  
 BRAUN, H. & TEICHMANN, E., 1912, *Versuche zur Immunisierung gegen Trypanosomen*, Jena.  
 BREINL, A. & NIERENSTEIN, M., 1909, *Ann. Trop. Med. Parasit.*, **3**, 395.  
 BROWN, W. H. & PEARCE L., 1920, *J. Exp. Med.*, **32**, 445.  
 BROWNING, C. H., 1907, *Brit. Med. J.*, ii, 1405; 1908, *J. Path. Bact.*, **12**, 166; 1927, *Brit. Med. J.*, ii, 978.  
 BROWNING, C. H., COHEN, J. B., ELLINGWORTH, S. & GULBRANSEN, R., 1926, *Proc. Roy. Soc., B.* **100**, 293; 1928, *ibid.*, **103**, 404; 1929, *ibid.*, B. **105**, 99.  
 BROWNING, C. H., COHEN, J. B., GAUNT, R. & GULBRANSEN, R., 1922, *Proc. Roy. Soc., B.* **98**, 329.

BROWNING, C. H., COHEN, J. B. & GULBRANSEN, R., 1922, *Brit. Med. J.*, i, 514.  
 BROWNING, C. H. & GULBRANSEN, R., 1918, *Proc. Roy. Soc. B.* **90**, 136; 1922,  
*J. Path. Bact.*, **25**, 395; 1925, *Brit. Med. J.*, i, 688; 1927, *J. Path. Bact.*, **30**,  
 513; 1928<sup>1</sup>, *J. Pharmacol.* **34**, 187; 1928<sup>2</sup>, *J. Path. Bact.*, **31**, 134.  
 BROWNING, C. H., GULBRANSEN, R. & KENNAWAY, E. L., 1919, *J. Path. Bact.*,  
**23**, 106.  
 BROWNING, C. H., GULBRANSEN, R., KENNAWAY, E. L. & THORNTON, L. H. D.,  
 1917, *Brit. Med. J.*, **1**, 73.  
 BROWNING, C. H., GULBRANSEN, R. & THORNTON, L. H. D., 1917, *Brit. Med. J.*, ii, 70.  
 BRUSSIN, A. M., 1929, *Z. ImmunForsch.*, **62**, 424.  
 BRUSSIN, A. M. & BELETZKY, W. K., 1925, *Zbl. Bakt.*, Abt. I, Orig., **96**, 32.  
 BUSCHKE, A. & KROÓ, H., 1923, *Klin. Wschr.*, **2**, 580.  
 BUSCK, G., 1906, *Biochem. Z.*, **1**, 425.  
 CAPPELL, D. F., 1929, *J. Path. Bact.*, **32**, 629; 1930, *ibid.*, **33**, 429.  
 CASTELLI, G., 1913, *Z. Chemotheer.*, **1**, 122.  
 CHESNEY, A. M., HALLEY, C. R. L. & KEMP, J. E., 1927, *J. Exp. Med.*, **46**, 223.  
 CHESNEY, A. M. & KEMP, J. E., 1924, *J. Exp. Med.*, **39**, 553; 1925, *ibid.*, **42**, 17;  
 1926, *ibid.*, **44**, 589.  
 COLLIER, W. A., 1929, *Z. Hyg. InfektKr.*, **109**, 577.  
 COLLIER, W. A. & BERNHAGEN, I., 1928, *Z. Hyg. InfektKr.*, **109**, 383.  
 COLLIER, W. A. & KRAUSE, M., 1929, *Z. Hyg. InfektKr.*, **110**, 522.  
 CUNNINGHAM, J., 1925, *Trans. Soc. Trop. Med. Hyg.*, Lond., **19**, 11.  
 DALE, H. H. & DOBELL, C., 1917, *J. Pharmacol.*, **10**, 399.  
 DOBELL, C. & LAIDLAW, P. P., 1926, *Parasitology*, **18**, 206.  
 DUKE, L., 1927, *League of Nations International Commission on Human Trypano-*  
*somiasis*, Interim rep. C.H. 536, p. 24, Geneva.  
 DURHAM, F. M., GADDUM, J. H. & MARCHAL, J. E., 1929, *Sp. Rep. Ser. Med. Res.*  
*Coun.*, Lond., No. **128**.  
 DURHAM, F. M., MARCHAL, J. & KING, H., 1926, *J. Pharmacol.*, **28**, 341.  
 EHRLICH, P., 1907, *Berl. klin. Wschr.*, **44**, 233, 341; 1909<sup>1</sup>, *Ber. deuts. chem. Ges.*,  
**42**, 17; 1909<sup>2</sup>, *Münch. Med. Wschr.*, **56**, 217; 1909<sup>3</sup>, *Arch. Schiffs- u. Tropenhyg.*,  
**13**, Beiheft **6**, 91.  
 EHRLICH, P. & GUTTMANN, P., 1891, *Berl. klin. Wschr.*, **28**, 953.  
 EHRLICH, P. & HATA, S., 1910, *Die experimentelle Chemotheerapie d. Spirilloseen*, Berlin.  
 EHRLICH, P., ROEHL, W. & GULBRANSEN, R., 1909, *Z. ImmunForsch.*, Tl. I, Orig.,  
**3**, 296.  
 EHRLICH, P. & SHIGA, K., 1904, *Berl. klin. Wschr.*, **41**, 329, 362.  
 FEILER, M., 1920, *Z. ImmunForsch.*, Tl. I, Orig., **30**, 95.  
 FELDT, A. & EISENMAYER, C., 1928, *Z. Hyg. InfektKr.*, **109**, 410.  
 FELDT, A. & SCHOTT, A., 1927, *Z. Hyg. InfektKr.*, **107**, 453.  
 FLEMING, A., 1924, *Proc. Roy. Soc. B.* **98**, 171.  
 FOURNEAU, E., TRÉFOUEL, J. & VALLÉE, J., 1924, *Ann. Inst. Pasteur*, **38**, 81.  
 FRANKE, E., 1905, *Therapeutische Versuche bei Trypanosomenerkrankung*, Jena.  
 FREUND, R., 1925, *Z. ImmunForsch.*, **43**, 253.  
 GIEMSA, G., 1927, *Münch. med. Wschr.*, **74**, 574.  
 GONDER, R., 1911, *Zbl. Bakt.*, Abt. I, Orig., **61**, 102; 1912<sup>1</sup>, *ibid.*, Abt. I, Orig.,  
**62**, 168; 1912<sup>2</sup>, *Z. ImmunForsch.*, Tl. I, Orig., **15**, 257; 1914, *ibid.*, Tl. I,  
 Orig., **21**, 309.  
 GRAHAM, J. G., 1925, *Brit. Med. J.*, ii, 826.  
 GRAY, J. D. A., 1929, *Ann. Trop. Med. Parasit.*, **23**, 241, 273.  
 GUTTMANN, P., 1891, *Berl. klin. Wschr.*, **28**, 5.  
 HATA, S., 1910, see EHRLICH, P. & HATA, S., 1910.  
 HATCHER, R. A. & WEISS, S., 1926, *J. Pharmacol.*, **29**, 279.  
 HEGNER, R., SHAW, E. H. & MANWELL, R. D., 1928, *Amer. J. Hyg.*, **8**, 564.  
 HEWITT, L. F. & KING, H., 1926, *J. Chem. Soc.*, Lond., **1**, 817.  
 HEWITT, L. F., KING, H. & MURCH, W. O., 1926, *J. Chem. Soc.*, Lond., **1**, 1355.  
 JANCSÓ, N., 1918, *Zbl. Bakt.*, Abt. I, Orig., **81**, 457.  
 JOLLOS, V., 1924, *Zbl. Bakt.*, Abt. I, Orig., **93**, 22\*.  
 JUNGEBLUT, C. W., 1927, *Z. Hyg. InfektKr.*, **107**, 357.  
 KIKUTH, W. & REGENDANZ, P., 1929, *Z. ImmunForsch.*, **61**, 422.  
 KING, H., 1927, *J. Chem. Soc.*, Lond., **1**, 1049.

KING, H. & MURCH, W. O., 1924, *J. Chem. Soc., Lond.*, **125**, 2595; 1925, *ibid.*, **127**, 2632.

KOCH, R., 1881, *Mitt. GesundhAmt.*, **1**, 234; 1890, see *Gesammelte Werke*, 1912, Leipzig, **1**, 650.

KOLLE, W., 1924, *Deuts. med. Wschr.*, **50**, 1235.

KOPANARIS, P., 1911, *Arch. Schiffs- u. Tropenhyg.*, **15**, 586.

KRANTZ, W., 1926, *Z. ImmunForsch.*, **48**, 207.

KRITSCHEWSKI, I. L., 1927, *Zbl. Bakt., Abt. I, Orig.*, **104**, 214\*; 1928, *Z. ImmunForsch.*, **59**, **1**.

KRITSCHEWSKI, I. L., BASKIN, M. M. & LEBEDJEVA, M. N., 1929, *Z. ImmunForsch.*, **64**, 382.

KRITSCHEWSKI, I. L. & BRUSSIN, A. M., 1927, *Z. ImmunForsch.*, **49**, 180.

KRITSCHEWSKI, I. L. & KAGANOVA, S. S., 1929, *Z. ImmunForsch.*, **61**, 478.

KRITSCHEWSKI, I. L. & MEERSOHN, I. S., 1926, *Z. ImmunForsch.*, **47**, 407.

KROÓ, H. & MANO, Y., 1927, *Deuts. med. Wschr.*, **53**, 603.

KUDICKE, R., 1911, *Zbl. Bakt., Abt. I, Orig.*, **59**, 182; 1912, *ibid.*, Abt. I, Orig., **61**, 113.

KUDICKE, R., FELDT, A. & COLLIER, W. A., 1924, *Z. Hyg. InfektKr.*, **102**, 135.

LAIDLAW, P. P., DOBELL, C. & BISHOP, A., 1928, *Parasitology*, **20**, 207.

LAVERAN, A., 1902, *C.R. Acad. Sci., Paris*, **134**, 735; 1904, *ibid.*, **139**, 19; 1905, *ibid.*, **140**, 287, 1081.

LAVERAN, A. & MESNIL, F., 1912, *Trypanosomes et Trypanosomiasis*, 2nd Edn., Paris.

LAVERAN, A. & ROUDSKY, D., 1912, *C.R. Soc. Biol., Paris*, **72**, 313.

LEUPOLD, F., 1924, *Arb. Inst. exp. Ther. Frankfurt*, H. **17**, 19; 1925, *Z. Hyg. InfektKr.*, **104**, 641; 1928, *Arb. Inst. exp. Ther. Frankfurt*, H. **21**, 110.

LEVADITI, C. & ROCHE, J., 1907, *C.R. Soc. Biol., Paris*, **62**, 815.

LEVADITI, C. & YAMANOUCHI, T., 1908, *C.R. Soc. Biol., Paris*, **65**, 23.

LEWY, F., 1925, *Z. ImmunForsch.*, **48**, 196, 243.

LEWY, F. & GUREWITSCH, M., 1926, *Z. Hyg. InfektKr.*, **106**, 532.

LISGUNOVA, A. W., 1928, *Z. ImmunForsch.*, **57**, 292.

LISTER, J., 1867<sup>1</sup>, *Lancet, Lond.*, i, 326, 357, 387, 507; 1867<sup>2</sup>, *ibid.*, ii, 95; 1909, see *Collected Papers*, Oxford, **2**, i.

MACKIE, T. J., 1925, *J. Hyg., Camb.*, **24**, 176.

MADSEN, T. & MÖRCH, J. R., 1926, *Acta Tuberc. Scand.*, **2**, 99; 1928, *Z. Hyg. InfektKr.*, **109**, 224.

MANTEUFEL, 1908, *Arb. GesundhAmt., Berl.*, **27**, 327.

MARGULIES, M., 1910, *Deuts. med. Wschr.*, **36**, 1907.

MARKS, L. H., 1914, *Berl. klin. Wschr.*, **51**, 1886.

MELENNEY, H. E., 1928, *J. Exp. Med.*, **48**, 65.

MESNIL, F. & BRIMONT, E., 1908, *Ann. Inst. Pasteur*, **22**, 856.

MOLDOVAN, J., 1914, *Z. ImmunForsch.*, Tl. I, Orig., **21**, 481.

MÖLLGAARD, H., 1924, *Chemotherapy of Tuberculosis*, Copenhagen.

MORGENROTH, J., 1918, *Deuts. med. Wschr.*, **44**, 961, 988; 1919, *ibid.*, **45**, 505; 1924, *Zbl. Bakt., Abt. I, Orig.*, **93**, 94.\*

MORGENROTH, J. & FREUND, R., 1924, *Klin. Wschr.*, **8**, 53.

MORGENROTH, J. & KAUFMANN, M., 1912, *Z. ImmunForsch.*, Tl. I, Orig., **15**, 610.

MORGENROTH, J. & LEVY, R., 1911, *Berl. klin. Wschr.*, **48**, 1560, 1979.

MORGENROTH, J. & ROSENTHAL, F., 1911, *Z. Hyg. InfektKr.*, **68**, 418, 506.

MORGENROTH, J. & WRESCHNER, H., 1923, *Deuts. med. Wschr.*, **49**, 1322.

MUELLER, J. H., 1919, *J. Path. Bact.*, **22**, 308.

MÜHLENS, P., 1929, *Arch. Schiffs- u. Tropenhyg.*, **33**, Beiheft **3**, 44.

MÜHLENS, P. & KIRSCHBAUM, W., 1924, *Arch. Schiffs- u. Tropenhyg.*, **28**, 131.

MÜHLENS, P., WEYGANDT, W. & KIRSCHBAUM, W., 1920, *Münch. med. Wschr.*, **67**, 831.

NEISSER, A., 1911, *Arb. GesundhAmt., Berl.*, **37**.

NEUMANN, R., 1911, *Z. Hyg. InfektKr.*, **69**, 100.

NEVEN, O., 1909, *Ueber d. Wirkungsweise d. Arzneimittel b. Trypanosomiasis*, Giessen.

NOTTHHAAS, R., 1927, *Münch. med. Wschr.*, **74**, 761.

NUTTALL, G. H. & HADWEN, S., 1909, *Parasitology*, **2**, 156, 236.

OEHLER, R., 1914, *Z. Hyg. InfektKr.*, **77**, 356.

OELZE, F. W., 1928, *Arb. Inst. f. exp. Ther. Frankfurt*, H. **21**, 150.

ÖRSKOV, J. & SCHMIDT, A., 1928, *Z. ImmunForsch.*, **55**, 69.

PERUZZI, M., 1928, *League of Nations International Commission on Human Trypanosomiasis*, Final rep. C.H. 629, p. 245, Geneva.

PLEHN, A., 1928, *Z. Hyg. InfektKr.*, **108**, 685.

PRIGGE, R. & ROTHERMUNDT, M., 1928, *Z. Hyg. InfektKr.*, **108**, 399.

REICHENOW, E. & REGENDANZ, P., 1927, *Abh. Ausl. dsk.*, Hamburg Universität, **26**; Reihe D. Medizin **2**, 446.

REINER, L. & KÖVESKUTY, J., 1927, *Deuts. med. Wschr.*, **53**, 1988.

RICHARDSON, U. F., 1928, *J. Comp. Path.*, **41**, 199.

RIECKENBERG, H., 1917, *Z. ImmunForsch.*, Tl. I, Orig., **26**, 53.

RITZ, H., 1914, *Deuts. med. Wschr.*, **40**, 1355; 1916, *Arch. Schiffs- u. Tropenhyg.*, **20**, 397.

ROBERTSON, M., 1929, *Parasitology*, **21**, 375.

ROEHL, W., 1909<sup>1</sup>, *Berl. klin. Wschr.*, **46**, 494; 1909<sup>2</sup>, *Z. ImmunForsch.*, Tl. I, Orig., **1**, 633; 1926<sup>1</sup>, *Arch. Schiffs- u. Tropenhyg.*, **30**, Beiheft 1, 103; 1926<sup>2</sup>, *ibid.*, **30**, Beiheft 3, 11; 1926<sup>3</sup>, *Deuts. med. Wschr.*, **52**, 2017; 1927, *Arch. Schiffs- u. Tropenhyg.*, **31**, Beiheft 1, 48; 1929, *Ind. Med. Gaz.*, **64**, 563.

RONA, P. & BLOCH, E., 1921, *Biochem. Z.*, **121**, 235.

ROSENTHAL, F., 1913, *Z. Hyg. InfektKr.*, **74**, 489.

ROTHERMUNDT, M. & DALE, J., 1911, *Deuts. med. Wschr.*, **37**, 1790.

RUBINSTEIN, P. L., 1928, *Z. ImmunForsch.*, **57**, 107.

SAZERAC, R., NAKAMURA, H. & KITCHEVATZ, M., 1927, *C.R. Acad. Sci.*, Paris, **184**, 411.

SCHIEMANN, O., 1923, *Z. Hyg. InfektKr.*, **97**, 280.

SCHILLING, C., 1927, *Handbuch d. path. Mikroorg.*, hrsg. Kolle, Kraus u. Uhlenhuth, Jena, **8**, 95.

SCHLOSSBERGER, H., 1928, *Z. Hyg. InfektKr.*, **108**, 627; 1929, *Zbl. Bakt.*, Abt. I, Orig., **110**, 210.\*

SCHNITZER, R., 1926, *Z. ImmunForsch.*, **47**, 116.

SCHNITZER, R. & ROSENBERG, E., 1926, *Z. ImmunForsch.*, **48**, 23; 1927, *ibid.*, **49**, 393.

SCHNITZER, R. & SILBERSTEIN, W., 1927<sup>1</sup>, *Z. ImmunForsch.*, **49**, 387; 1927<sup>2</sup>, *ibid.*, **49**, 551; 1927<sup>3</sup>, *ibid.*, **53**, 439; 1928, *ibid.*, **58**, 159.

SEI, S., 1923, *Z. Hyg. InfektKr.*, **100**, 416.

SERGENT, E. & E., 1921, *Ann. Inst. Pasteur*, **35**, 125; 1921<sup>2</sup>, *Arch. d. Inst. Past. de l'Afrique du Nord*, **1**, 1.

SILBERSTEIN, W., 1927, *Z. ImmunForsch.*, **54**, 324.

STEFFAN, P., 1922, *Z. Hyg. InfektKr.*, **98**, 263.

STILLING, J., 1891, *Lancet*, Lond., i, 872.

TALIAFERRO, W. H. & TALIAFERRO, L. G., 1929, *J. Prevent. Med.*, **3**, 209.

TEICHMANN, E., 1916, *Z. Hyg. InfektKr.*, **82**, 511.

TERRY, B. T., 1911, *Monog. Rockefeller Inst. Med. Res.*, No. **3**.

TSUZUKI, M., 1911, *Z. Hyg. InfektKr.*, **68**, 364.

TUBBY, A. H., FERGUSON, A. R., MACKIE, T. J. & HIRST, L. F., 1919, *Lancet*, Lond., i, 838.

UHLENHUTH & GROSSMANN, 1927, *Zbl. Bakt.*, Abt. I, Orig., **104**, 166.\*

UHLENHUTH & SEIFFERT, W., 1928, *Med. Klinik*, **24**, 584.

VOEGTLIN, C., 1929, *J. Pharmacol.*, **35**, 189.

VOEGTLIN, C., DYER, H. A. & MILLER, D. W., 1924, *J. Pharmacol.*, **23**, 55.

VOEGTLIN, C. & SMITH, H. S., 1920, *J. Pharmacol.*, **15**, 453, 475.

WALBUM, L. E., 1921, *C.R. Soc. Biol.*, Paris, **85**, 761; 1925, *Z. ImmunForsch.*, **43**, 433; 1926, *ibid.*, **47**, 213; 1927, *Z. Tuberk.*, **48**, 193; 1928, *ibid.*, **51**, 209; 1929<sup>1</sup>, *ibid.*, **53**, 292; 1929<sup>2</sup>, *Z. ImmunForsch.*, **61**, 146.

WALBUM, L. E. & MÖRCH, J. R., 1923, *Ann. Inst. Pasteur*, **37**, 396.

WARTHIN, A. S., 1916, *Amer. J. Med. Sci.*, **152**, 508; 1918, *Amer. J. Syph.*, **2**, 425; 1929, *Brit. Med. J.*, ii, 236.

WERBITZKI, F. W., 1910, *Zbl. Bakt.*, Abt. I, Orig., **53**, 303.

WRIGHT, A. E., 1927, *Lancet*, Lond., ii, 1327.

YORKE, W., 1925, *Trans. Soc. Trop. Med. Hyg.*, **19**, 108.

YORKE, W., ADAMS, A. R. D. & MURGATROYD, F., 1930, *Ann. Trop. Med. Parasit.*, **24**, 115.

YORKE, W. & MACFIE, J. W. S., 1924, *Trans. Soc. Trop. Med. Hyg.*, Lond., **18**, 13.

Copies of this volume and of other volumes of the "System of Bacteriology" may be purchased directly from H.M. STATIONERY OFFICE at the following addresses :—ADASTRAL HOUSE, KINGSWAY, LONDON, W.C.2 ; 120, GEORGE STREET, EDINBURGH ; YORK STREET, MANCHESTER ; 1, ST. ANDREW'S CRESCENT, CARDIFF ; 15, DONEGALL SQUARE WEST, BELFAST ; or through any Bookseller.

#### *ABROAD.*

*Copies of Government Publications are obtainable through the following :—*

**AUSTRALIA.**—Messrs. Angus & Robertson, Ltd., 89, Castlereagh Street, Sydney, N.S.W. ; Messrs. Albert & Son, Ltd., 180, Murray Street, Perth, Western Australia ; Messrs. Oldham, Beddome & Meredith (1932) Pty., Ltd., 36, Elizabeth Street, and 96, Collins Street, Hobart, Tasmania.

**CANADA.**—The Imperial News Co., Ltd., 235, Fort Street, Winnipeg ; 517, Burrard Street, Vancouver ; The William Dawson Subscription Service, Ltd., 70, King Street East, Toronto, Ontario.

**DENMARK.**—A. Busck, Kjobmagergade, 49, Copenhagen.

**FINLAND.**—Akademiska Bokhandeln, Helsingfors.

**GERMANY.**—Messrs. A. Asher & Co., Behrenstrasse 17, Berlin, W.8.

**GOLD COAST.**—Wesleyan Methodist Book Depot, P.O. Box 100, Cape Coast ; branches at Accra, Kumasi and Sekondi.

**HOLLAND.**—N. V. Martinus Nijhoff' Boekhandel, Lange Voorhout 9, 's-Gravenhage.

**INDIA.**—Messrs. Thacker & Co., Ltd., Bombay ; Messrs. Higginbothams, Ltd., Madras and Bangalore.

**IRISH FREE STATE.**—Messrs. Eason & Son, Ltd., 40-41, Lr. O'Connell Street, Dublin.

**JAPAN.**—Maruzen Company Ltd., 6, Nihonbashi Tori-Nichome, Tokyo.

**NEW ZEALAND.**—Messrs. Whitcombe & Tombs, Ltd., Auckland, Christchurch, Dunedin and Wellington.

**NORWAY.**—Cammermeyers Boghandel, Karl Johans Gate 41-43, Oslo.

**SOUTH AFRICA.**—The Central News Agency, Ltd., P.O. Box 1033, Johannesburg ; P.O. Box 9, Cape Town ; P.O. Box 938, Durban ; P.O. Box 356, Port Elizabeth ; Pretoria, Transvaal..

**SWEDEN.**—A-B. C.E. Fritzes Kungl. Hofbokhandel, Fredsgatan 2, Stockholm.

**U.S.A.**—The British Library of Information, 270, Madison Avenue, New York.



616.01

DATE OF ISSUE

S989

This book must be returned within 3/7/14-6  
days of its issue. A fine of ONE ANNA per day  
will be charged if the book is overdue.

~~RECEIVED~~

~~STAMP~~

Class No. 616-01 Book No. 59  
Vol 6

**Author.....**

Title. System of Bacteriology  
in Relation to Medicine Acc No. 59199

<u>Prairie Leaf Sete:</u>	
<del>889575890</del>	
<del>Vidya</del>	<del>1713.50</del>